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TROUT HEPATOMA RESEARCH CONFERENCE PAPERS

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RESEARCH REPORT 70

RESEARCH REPORTS

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TROUT HEPATOMA RESEARCH CONFERENCE PAPERS

Edited by
John E. Halver and
Ian A. Mitchell

RESEARCH REPORT 70

UNITED STATES DEPARTMENT OF THE INTERIOR
Stewart L. Udall, *Secretary*
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FISH AND WILDLIFE SERVICE
Clarence F. Pautzke, *Commissioner*
BUREAU OF SPORT FISHERIES AND WILDLIFE
John S. Gottschalk, *Director*

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FOREWORD

These papers of the Third National Trout Hepatoma Research Conference held in the Interior Building, Portland, Oreg., March 18 and 19, 1965, are arranged in order of presentation and are reproduced in the form received. The results, discussions, and opinions expressed are those of the individual or group represented and do not necessarily reflect the views of either the National Cancer Institute or the Department of the Interior. This research conference was sponsored by the Field Studies Branch of the National Cancer Institute and by the Division of Fishery Research of the Bureau of Sport Fisheries and Wildlife.

The purpose of this conference was to review the status of research on rainbow trout hepatoma, to arrange research discussions between the major active research groups in this field, and to project probable productive research programs for future efforts.

The conference scheduled presentation of highlights from the research efforts of each participant which served as a stimulus for open discussion. Therefore, this research report is arranged in order of: conference workbook material; individual presentation; and then open discussion. The report closes with a succinct summary and challenging prospectus by Dr. Paul Kotin, Scientific Director for Etiology, National Cancer Institute.

The first two conferences were preliminary and exploratory and the proceedings of those will not be published.

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FRONTISPIECE

(FOUR COLOR PLATES)

COLOR PLATE 1.

a. Normal Rainbow trout liver with spatula under lower lobe. The uniform reddish brown color is indicative of a healthy liver.

b. Histology of normal rainbow trout liver showing liver cords (muralia) averaging two liver cells in width and alternating with parent blood sinusoids in which red blood cells may be seen. Iron hematoxylin and orange G. × 650.

COLOR PLATE 2.

a. Multinodular hepatomatous trout liver in which 12 or more rounded, typical tumors may be seen. × 1.

b. Histological detail of advanced hepatoma showing the trabecular pattern on the right, with collapsed sinusoids, avascularity, increased nuclearity, occasional bizarre liver cells, and occasional mitoses. On the left is an area showing fibrotic replacement of degenerating liver cells, some of which are spindle-shaped, indicating a malignant tendency. I.H. and O.G. × 650.

COLOR PLATE 3.

a. Two large advanced yellowish hepatomas in liver above center of probe. Tip of probe points to metastasis of hepatoma to kidney (swollen area). × 1.

b. Histology of trout hepatoma which had metastasized to eight different organs. Note frequency of bizarre liver cells, atypical mitotic figures, and greatly altered liver architecture. H. and E. × 1600.

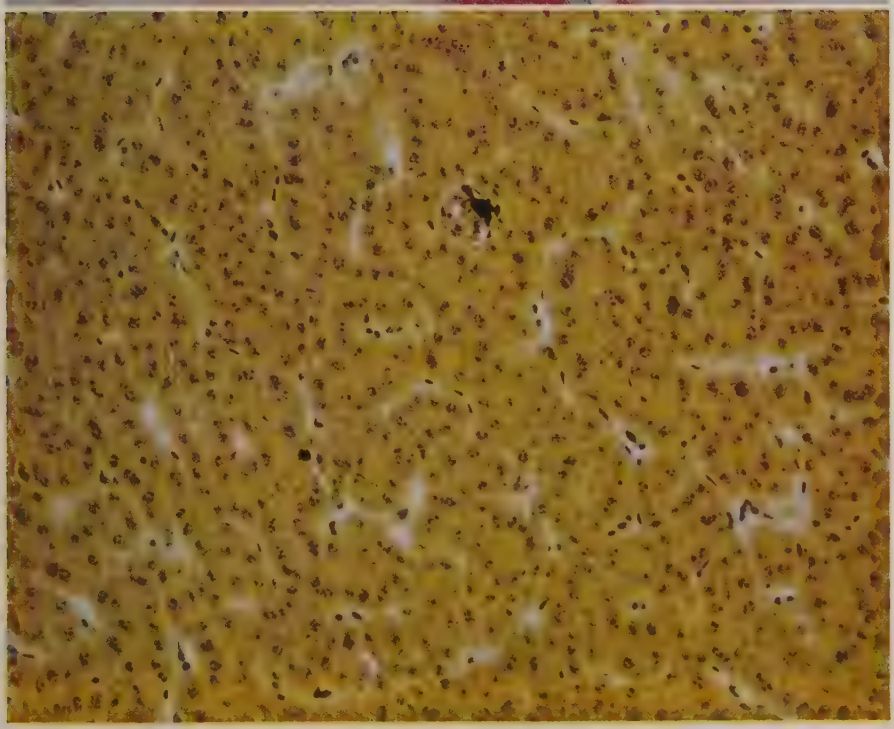
COLOR PLATE 4.

a. Juvenile trout force-fed 1 mg/kg body weight of crude aflatoxin and sacrificed 8 days later, showing severe liver necrosis (white area) and hyperemic, prenecrotic liver (reddish portion). Hemorrhagic visceral fat appears between pyloric caeca (above tip of hemostat) and along gastrointestinal tract. × 1.

b. Liver histology of trout force-fed crude aflatoxin, showing pale necrotic tissue generously stippled with nucleated red blood cells. A prenecrotic, somewhat hyperemic area is also present (upper right). H. and E. × 650.

Color Plate 1

a



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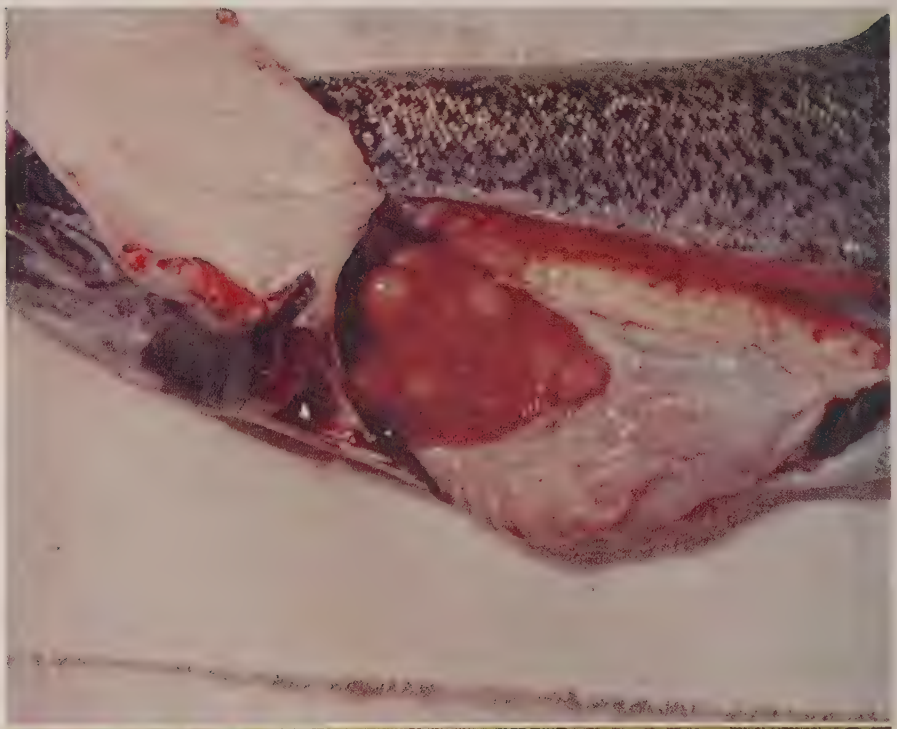

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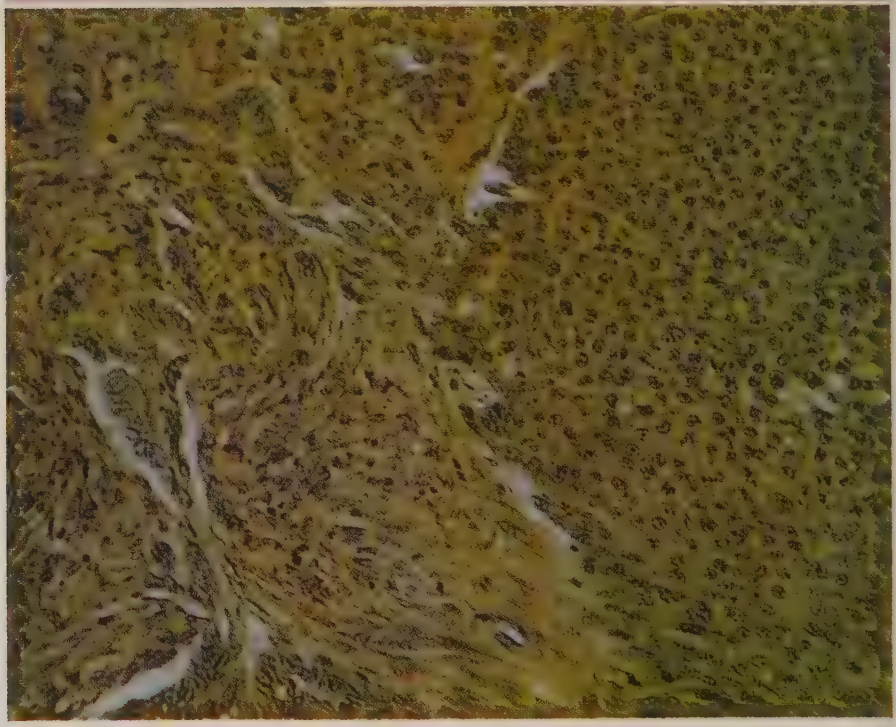

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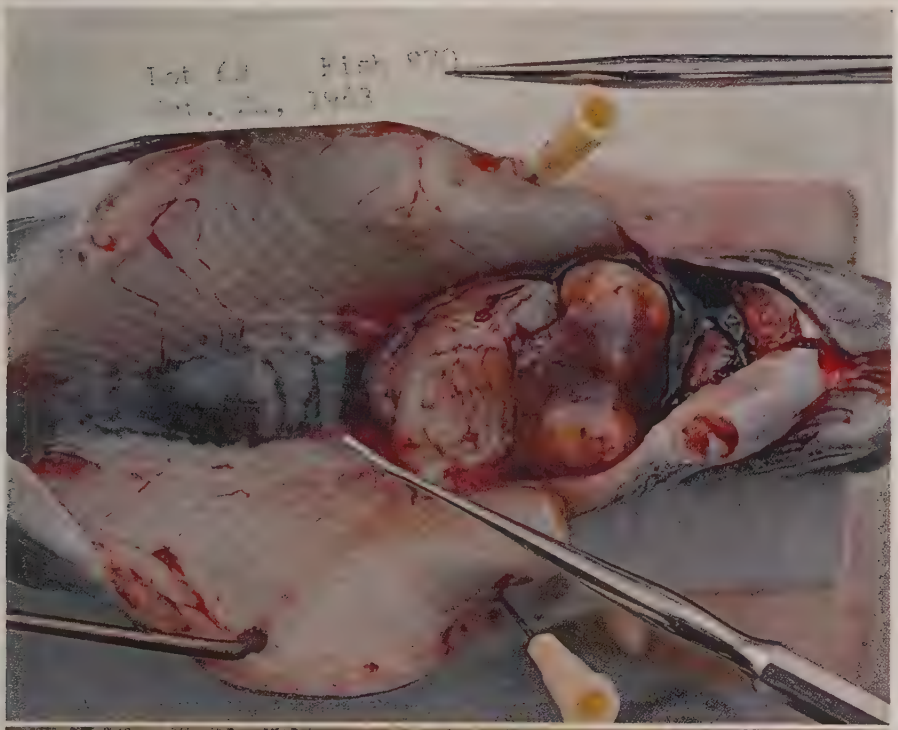

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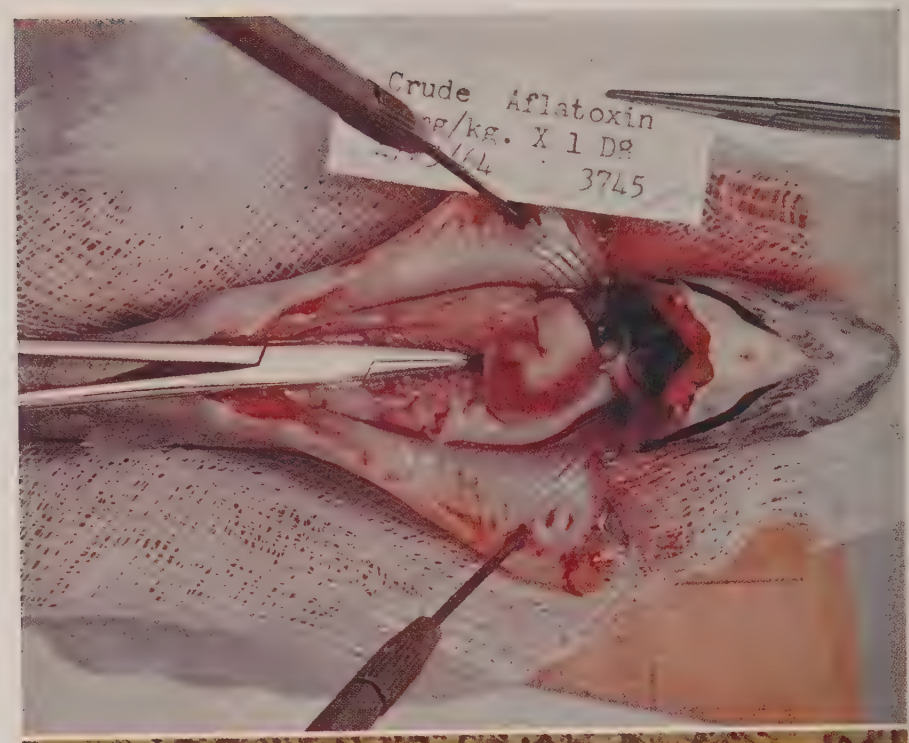

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ETIOLOGY AND EPIDEMIOLOGY OF TROUT HEPATOMA

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Occurrence of trout hepatoma has been reviewed extensively. It is apparent that the disease has occurred in trout in as widespread locations as France, Germany, Italy, Africa, and the eastern, midwestern, and western areas of the United States. When the outbreak in the United States was recognized as epidemic, meetings were held to review the problem, and a number of research programs evolved. At the outset of these studies there was no reasonable chance to determine the complete feeding or management history of those fish known to have developed the disease.

As trout culture has become more intensive and as the end use of trout has changed from the planting of fry in streams, lakes, and ponds to the production of larger fish for planting or market, the whole trout culture program has undergone change. Selection and domestication of trout strains have become more intensive. There have been great changes in the types of food available from essentially fresh materials to dry, pelleted, commercially prepared feeds. Disease treatment has undergone a great deal of change, mostly on the basis of practical experience. There has been a great increase in the transportation of trout and trout eggs between different locations within States and between States, resulting in the possible transfer of disease from one location to another. To make the problem more difficult, most of these changes in trout culture methods have taken place without adequate documentation of the reasons for change or the results achieved.

RAINBOW TROUT STRAINS

At present, rainbow trout is a species of great importance to fish culturists in the United States, Europe, Japan, and to a lesser extent in other areas. This species has been raised under domestic conditions for the past hundred years, and as a consequence the genetic history has become confused. The original stocks which form the basis of modern rainbow trout culture probably originated from the McCloud River watershed in California. It is likely that various isolated strains of rainbow trout have been interbred and, further, the rainbow trout has been interbred with closely related species, such as cutthroat trout. Today trout eggs are obtained from private breeders or from State or Federal brood-stock stations. Only rarely are eggs obtained from wild strains in natural lakes, streams, or reservoirs. As a

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consequence of this more intensified culture, the individual trout breeder has imposed artificial pressure upon the natural pressures causing selection of variants from the species. These selection pressures are directed to developing strains that have specific morphological characteristics, rates of growth, behavior patterns, and, ultimately, size. This management of the trout strains is complicated by the effects of environment and food. It is quite evident that the results confirm that the rainbow trout is an extremely "plastic" species.

CULTURE CONDITIONS

In general, for proper development of the ova, eggs must be obtained from brood fish cultured in waters where the temperatures are less than 56° F. It is quite evident that ova taken from brood stocks cultured at moderate temperatures will prove satisfactory under very extreme conditions. For example, trout eggs taken from brood fish where the temperature never exceeds 56° F. will, after hatching, grow well when cultured in water where the temperature is never less than 58° F. Similarly, offspring can be raised at much lower temperatures than the brood fish from which eggs have been obtained. Under high temperature conditions, the growth is rapid, and disease, when present, will develop rapidly and will be difficult to control. At very low temperatures, the fish grow very slowly, since they fail to accept food readily and, as a consequence, become weak and often succumb to diseases not evident at higher temperatures.

DISEASE CONTROL

A wide variety of chemicals has been used for disease treatment. These include formaldehyde, copper sulfate, malachite green, and mercurial compounds, as well as the more exotic drugs, including "sulfa-type" drugs and antibiotics. The effect of each of these drugs was never fully evaluated. They were tested and found to cause a remission of a disease and were then adopted widely. In some cases, the drugs or chemicals gave widely different effects under different water conditions.

FOOD AND EPIDEMIOLOGY

The problems of fish culture have varied from year to year. However, an important disease was observed during 1960–61. This liver disease, hepatoma, was recognized in a wide variety of locations. Previous outbreaks of a similar disease have been reported, but these were limited to a few locations and failed to create any significant long-term interest. The outbreak in 1960–61 appeared to have as a common factor the introduction of mixed dry feeds, either fed as pellets or admixed to wet feeds. There was no clear indication of exact composition of the diets, their vitamin or mineral fortification, or use of additives for disease treatment.

Another disease causing hepatic change was recognized in Europe and Africa. The symptoms of that disease were different in many respects, but most notably in the presence of ceroid pigment in the livers of the affected fish. The disease in the United States, where dry feeds were fed, appeared to develop in outwardly normal appearing fish. Liver histology in the case of trout hepatoma in this case generally lacks ceroid pigment even though the nodules have characteristics resembling malignancy. In some rare cases metastases were found in the kidneys, gills, and other tissues.

In Europe, liver lipoid degeneration (L.L.D.) was the predominant disease. The tumors were found at a later stage in those fish which had recovered or possibly had been unaffected by L.L.D.

Early information suggested that dry feeds were not involved since only fresh food materials had been fed to fish developing the L.L.D. The therapeutic measures used to treat L.L.D. were varied and included the addition of dry ingredients to the wet foods. Frequently, feeding histories failed to provide complete data. This was partly due to a reluctance on the part of a fish culturist to admit to a disease, or to normal management practices or, as was often the case, incomplete records.

The general interest in hepatoma in trout has included European, Australian, and African fish culturists. In Europe, liver lipoid degeneration was described many years ago. However, it was not until approximately 8 years ago that liver tumors were noted along with this disease. The most important outbreak was reported from Italy, although a similar outbreak was found in trout in hatcheries in France.

In France, in Germany, and in England, there has been a gradual introduction of dry pelleted rations. Dry components have been incorporated into so-called wet rations, and pelleted rations have been introduced. It was obvious that L.L.D. and a wide variety of bacterial diseases were found where fish were fed wet rations which consist of fish from the Atlantic or Mediterranean. These many diseases and poor survival are undoubtedly associated with the poor quality of the food, which results largely from spoilage due to a lack or absence of refrigeration.

In southern Germany the wet materials are preserved by heat pasteurization and then mixed with dry components, which include fish meal, shrimp and crab meats, wheat middlings, oat middlings, and variable amounts of vitamin supplements. One interesting development can be noted, and that is the production of a successful commercial dry pellet which consists of less than 30 percent protein. The vitamin supplement in this ration is similar to those used in this country. Fish raised for 15 months on this ration were free of disease, including liver disease.

In France dry rations are produced, but until very recently they have not been received with enthusiasm. The principal diets in France have been fishery products which generally have not been refrigerated and usually are spoiled. The major difficulties in the French diets would be oxidation of food components or formation of toxic products through spoilage from bacterial contamination, and thus the fish in these hatcheries suffer a high mortality. It is quite evident where fresh foods of good quality have been used that disease is minimal and survival is excellent. The food components are varied, and there is very little consistent pattern to the incorporation of dry components. Here, again, the principal dry ingredients are wheat middlings, groundnut, cottonseed meals, or soybean meals, a variety of fish meals, brewer's yeast and fish solubles, added to fresh or spoiled wet components. Essentially half the dry matter in these rations is supplied by the dry solids from the wet components, and the remainder by the addition of dry meals. It would be difficult to determine the specific ingredient or ingredients that would induce a tumor, but certainly the overall poor quality of the "fresh" ingredients would be a contributory factor to high mortality, liver lipoid degeneration (L.L.D.), and possibly to tumor development.

In England and in Kenya, complete dry rations are available. These have had a variable degree of success. Generally they are not fed exclusively, but are combined with fresh liver and fish. Liver diseases have been noted in the past, but none were present in the fish

TABLE 1.—*Pelleted dry rations for trout—Foreign*

Ingredient	Percent in diet from—					
	England	Denmark	Holland	Germany	Kenya	
					No. 1, 1962	No. 2, 1963
Animal protein products:						
Meat and bone meal	10				10	10
Fish meal	20	15	30	5	30	30
Liver meal		10		12	20	20
Blood meal			6			
Dried skim milk	10	10		10		
Shrimp and crab meal				10		
Vegetable protein products:						
Soybean meal	5		6	3		
Sunflower meal			6			
Cottonseed meal		20			5	
Groundnut meal	10				15	
Cereal products:						
Maize meal			20.4			
Wheat meal			24			
Wheat middlings (pollard)	20			25	16.5	35.5
Wheat bran	10					
Wheat flour	7.5	24		25		
Other ingredients:						
Corn fermentation solubles				4		
Condensed fish solubles		12				
Brewer's yeast		2	6	4		
Torula yeast		4				
Cane molasses	2.5					
Tallow	1.25					
Salt		1			1	2
Minerals	2.5		.8			
Vitamins	1.25			2	2.5	2.5

TABLE 2.—*Pelleted dry rations for trout—Domestic*

Ingredient	Percent in diet from—								
	Colorado 1961	Minnesota pellet diet	Michigan 1961	Idaho 1963	New York 1960	Quebec 1956	Virginia		
							No. 1 1961–62	No. 2 1962	No. 3 1962–63
Animal protein products:									
Meat and bone meal				10		20			
Fish meal	16	10	36	31	24	10	18	30	40
Liver meal	15	12		5					
Dried skim milk	5	8	5		7	5	10	20	10
Whey product	5			10					
Vegetable protein products:									
Soybean meal		16		9		27.5		18	18
Cottonseed meal	16	16	22		15		24		
Alfalfa meal						7.5			
Cereal products:									
Wheat middlings		16	25	20		2.5	24	17	17
Wheat red dog	19.5				24				
Wheat germ meal						5			
Dried Brewer's grains						5			
Other ingredients:									
Distiller's solubles	10	10	1		17		12	10	10
Condensed fish solubles			1.5	0.5					
Brewer's yeast	10	10	4	5	10	5	10	5	5
Torula yeast			3						
Vitamin A & D feeding oil	2			3	3	0.4	2		
Kelp meal				3					
Salt	1	2	2	3		2.5			
Minerals						6.2			
Vitamins	0.5		0.5	0.5		3.4			

TABLE 3.—*Vitamin supplements*
[mg per 100 g final mix]

	Pelleted dry rations—foreign			Pelleted dry rations—domestic			
	England	Germany	Kenya	Colorado 1961	Idaho	Michigan	Quebec
Ascorbic acid		45			22.0		
Biotin					0.066		
Calcium panthothenate		5		4.4	5.5	22.0	
Choline chloride		14			27.6	550.0	
Folic acid		.8			0.33		
Inositol							
Menadione							
Niacin		10		13.2	11.0	22.0	
Para-aminobenzoic acid							
Pyridoxine.HCl		.5		0.88	2.2		
Riboflavin		1	25	2.2	9.9	10.0	3,400
Thiamine.HCl		.5		2.2	13.2		
Vitamin B_{12}				.003	.002	.03	
Vitamin A	330 I.U.	800 I.U.	2,540 I.U.	7.45 I.U.	7.45 I.U.		
Vitamin D	165 I.U.	160 I.U.	3,300 I.U.	0.99 I.U.	0.99 I.U.		
Vitamin E		3 I.U.			6.6 I.U.		
Alpha tocopherol							
Methionine				100.0			

of England examined at this time. In Kenya, rainbow trout frequently are found, at the time of sexual maturity, to suffer from a disease similar to both L.L.D. and hepatoma.

The overall feature in these various areas is the predominance of spoiled fresh foods and relatively high incidence of disease and mortality in the smaller fish (up to 200 g) which is followed by the appearance of tumor in the older survivors forming the brood stock. A wide variety of dry ingredients has been fed during the life span of these various groups of fish (see tables 1, 2, 3, and 4). To identify a specific tumor-inducing agent under these conditions is virtually impossible.

TABLE 4.—*Hepatoma experiments—Growth and conversion, experimental data*

Hatchery	Food type	Temperature	g food / g gain	g protein / g gain	g animal protein / g gain
American Falls	High protein + High fish meal A	55°–58°	1.57	0.61	0.39
Mackay	Low protein + High fish meal A	52°	1.64	0.62	0.49
Hagerman	High protein + Low fish meal A	59°	1.86	0.77	0.49
Glenwood	Low protein + Low fish meal A	58°	1.64	0.65	0.50
Yakima	High protein + No cottonseed + High fish meal A	32°–72°	1.45	0.62	0.54
Springville	Low protein + Low fish meal A	53°	2.32	0.77	0.55
Spokane	High protein + No cottonseed + High fish meal A	50°	1.52	0.65	0.58
Lewistown	High protein + Low fish meal B	56°	2.78	1.12	0.71
Vancouver	High protein + No cottonseed + High fish meal A	49°–54°	1.79	0.86	0.74
Midway	Low protein + Low fish meal B	56°	3.66	1.43	0.89
Parkview	High fiber	49°	4.36	1.40	1.22

CULTURE OF RAINBOW TROUT IN AUSTRALIA

One of the more important questions in studying hepatoma in trout is to determine whether the disease occurs as a natural consequence of age or feeding conditions. To provide a satisfactory answer to this question requires examination of large-sized trout obtained from the wild or, alternatively, older trout maintained in hatcheries where fresh or frozen animal products are fed. An interesting group of trout satisfying the latter conditions was located at the Snob's Creek Hatchery in Australia (J. C. F. Wharton, personal communication). This stock of rainbow trout has been isolated from all other rainbow trout since its introduction in the 1890's. The diet for the life span of these trout has been almost exclusively fresh or frozen meats, and thus the histology of the tissues is of some considerable importance. Examination of sections obtained from trout which had died under the hatchery conditions were free of any evidence of hepatoma, but reflected the presence of an intercurrent bacterial infection. Later samples obtained from freshly killed adult brood fish and fish from a demonstration pond confirmed that no liver tumors were present in fish of about 10 years in age or younger.

The normal hatchery practices at Snob's Creek are reviewed briefly to provide an understanding of the variables.

Normally, brood fish are spawned once at 3 years of age and then discarded. However, over the last few years approximately 500 rainbow 4-year-olds are spawned a second time to collect egg yield data. The rainbow trout begin spawning in May and the spawning cycle reaches a peak in June–July. Older trout are maintained in a demonstration pond, and these include fish up to about 10 years of age and many are at least 15 pounds in weight. Frozen liver has been the major food fed these fish.

The standard feeding tables are used as a guide for calculation of food allowance. For the first month after the absorption of the yolk sac, fry are fed six times per day on finely ground ($\frac{1}{16}$ inch diameter holes in tissue mincer plate) beef heart. From the beginning of the second month of feeding until the fry are approximately half a gram in size (about 900 per pound) they are fed finely ground beef liver six times per day. The fish usually attain this size by the end of the third month of feeding.

When the fish attain the half-gram size, they are transferred to outside rearing units, where they are fed four times per day on a diet consisting of equal parts by weight of mash and finely ground beef liver. Present practice includes the addition of dry ingredients; however, prior to 6 years ago the diet consisted of only freshly ground meat. When the fish are about 5 months old, they are fed on moist pellets (47 percent beef liver, 25.5 percent meat meal, 18 percent wheat middlings, 1 percent salt, vitamin supplement, a coloring agent, and 7.5 percent water). The rate of feeding of small pellets is 4 times per day, 5 days per week. In addition to the pellets, ground fresh liver or heart is fed 4 times per day for 2 days per week.

For older fish and brood fish a larger size pellet is fed 5 days per week in addition to liver or heart for the other 2 days.

During the last 3 months prior to spawning, crayfish scrap (the head and hypodermis) is crushed and added to the diet of the brood fish at a rate of approximately 10 percent of the total diet. The addition of crayfish shell improves the color of the eggs.

The water supply, approximately 15 cubic feet per second, is at a mini-

mum during the summer period (November through February), which supports a production of approximately 80,000 pounds of fish. During the winter months (June through September), the amount of flow that can be diverted to the hatchery is increased. However, during this period there is an increase in silt and organic residues from the land. The water is low in solids and particularly deficient in calcium, this being about 3 ppm or less. The water is obtained from a stream and the temperature will range from a low in winter of 38°–40° F. to a high of about 65° F. in the summer. During the winter months, when the forest uplands are leached by heavy rainfall, the deficiency of calcium becomes a problem, since the amount of zinc increases to approximately 0.3 ppm. If prolonged exposure occurs, this level of zinc is toxic in the water deficient in calcium.

A variety of diseases are present of which the causes are unknown. "Blue sac" and "gold sac" diseases result in the loss of a small number of the rainbow trout fry. Another disease affecting the rainbow trout, which is called gray saddle, develops as a gray, necrotic area on either side of the dorsal fin. In recent years the brood stock have been affected by a bacterial disease which results in abscesses on the kidneys and occasionally on the liver. Samples of livers from trout affected by this disease are included in the sections examined. This disease probably results from the infection by bacteria tentatively identified as from the *Aeromonas* or *Pseudomonas* group.

The principal ingredients in the dry portion of the diets prepared at Snob's Creek include meat meal; pollard, which is equivalent to wheat middlings; salt; and Vitamin A and D_3 fortification. Fresh meat includes beef or sheep heart and beef liver. Frozen liver and heart is maintained in a freezer at 0° F. and is obtained from a slaughterhouse where the livers and hearts are held for an indefinite period in the frozen condition. Frozen crayfish meal, which includes the head and the hypodermis of crayfish, is added to the diet of the brood fish. This product is obtained from a crayfish-processing plant where the crayfish is boiled and the head portion removed. The scrap is frozen in large galvanized containers which are approximately 30 gallons in capacity. These crayfish parts are then shipped by truck to Snob's Creek, where the crayfish is removed from the containers, broken up, and laid out on shelves in a freezer operating at 0° F.

It is significant to note that wheat middlings, meat meal, and salt were the only significant dry additives and that the fresh products were held frozen at low temperatures. Tumors were absent in older fish, confirming that tumors, if they occur naturally, are very, very infrequent.

CULTURE OF RAINBOW TROUT IN KENYA

The regular importation of both rainbow and brown trout into Kenya began about 1930. Although most fingerlings from these ova were used to stock private and public waters, a few were retained in order to develop a brood stock. By 1963, the number of ova produced locally was 120,000, as compared with approximately 40,000 imported from foreign sources. An additional 40,000 brown trout ova are imported annually. On two occasions char ova were imported from the United States, but there was only limited distribution of this species.

The principal source of rainbow and brown trout ova has been the Surrey Trout Farm in the United Kingdom. Occasionally small numbers of ova were

imported from Jonkershoek Fish Hatchery in Stellenbosch, South Africa, the last being imported in 1961.

In 1962 a dry pellet food was prepared (C. E. P. Watson, personal communication). These pellets were produced by a local miller and consisted of pollard (wheat middlings), groundnut cake, cottonseed cake, fish meal, meat and bone meal, liver meal, salt, and fortified with vitamins and trace minerals. The composition of the feed is shown in table 1.

In 1963 a disease condition was observed and mortality increased. The developing brood stock were sluggish, dark in color, and had distended abdomens. The coelom was filled with a yellow fluid, and the livers were abnormal, blotchy in color, with prominent nodules. These symptoms are similar to those observed in liver lipoid disease (L.L.D.). Ceroid occurs in the livers of trout having this disease, and cellular changes characteristic of hepatoma are present in the nodular livers of trout surviving the acute form of L.L.D. When the disease was first observed, the amount of pellets fed was reduced and an increased amount of fresh meat was included in the feeding program. This remedy appeared to have no great beneficial effect upon the fish, and further investigation was required.

A detailed chemical examination (C. E. P. Watson, personal communication) of the dry pellets led to several possible explanations for the disease outbreak. The dry pellets had been stored at the hatchery for more than 6 months. The sample had a free fatty acid value of 31.4 percent. Such a high value would suggest that the feed was undergoing oxidation. The ingredients for the feed were produced locally. Liver meal, meat meal, and bone meal were produced by the K.M.C. Abattoir, Athi River, Kenya. The decorticated cottonseed cake (expeller's meal) and the fish meal, prepared from freshwater fish, were produced in Uganda. The decorticated groundnut cake, an expeller's meal, was from Tanganyika. The pollard (wheat middlings) was produced by the flour mills in Kenya. It is useful to note that no insecticides or other agricultural chemicals are used in the regions where the pellet ingredients are produced.

The results from the experiments in the United States suggested that cottonseed meal or other vegetable protein sources could contribute directly to the development of a liver tumor. As a consequence, the feed formula was revised, and this formula is shown in table 1. This formula has been fed for 1 year, and gross examination of the fish at the end of that time showed a distinct improvement in the survival. Of 1,127, 1962 spawners, 713 had died (63 percent mortality). Of the 1,754, 1963 spawners, 111 (or 7 percent) had died during the year. The 1963 group had been fed the new ration for 1 year, indicating that the disease had been controlled. Thirty-three of the fish grown during 1964 developed symptoms which may result in their mortality. These 33 may be part of the 1963 group, since 100 of the latter had been mixed in with the 1964 stock. The results of the changes upon the success of the brood stock program cannot be evaluated until January of 1966, at which time the fish will have been fed the experimental diet for 2 years.

STUDIES ON THE ETIOLOGY OF TROUT HEPATOMA

A number of problems have arisen during recent years with the more extensive use of any food supplements and dry pellets. These difficulties can be traced in part to poor choices of ingredient due to a lack of adequate practical information on the suitability of ingredients and levels of supplementation. The rather widespread use of

pellets has also changed management methods and culture techniques.

The staff at the College of Fisheries at the University of Washington planned a series of experiments to establish factors that may have contributed to the onset of the disease. The principal objectives of these experiments were as follows:

1. To evaluate the role of various food ingredients as factors in the induction of the tumor.
2. To determine the effects of various management practices such as amounts of food, rate of growth, and disease treatments on the occurrence of the tumor.
3. To determine whether particular environmental factors such as variation in the quality, quantity, and temperature of hatchery water supplies could influence the development of tumors.
4. To test the possibility that genetic differences were implicated in the induction of tumors.
5. To assess the possibility that the tumor could result from infection by organisms or viruses present in water supplies or transmitted from the adults through the eggs.

Preliminary information suggested that the disease was restricted to trout fed dry pellets. Review of the information available on food formulations identified the principal ingredients commonly present in suspected formulas as cottonseed meal, fish meal, wheat middlings, and distillers' solubles. These dry materials were chosen for detailed study, since they were the principal ingredients added to fresh or frozen fish, liver, spleen, or lungs and fed in Federal hatcheries where hepatoma was found.

Further consideration of the composition of trout diets which had been fed in hatcheries where tumor was found led to a general conclusion that those diets which contained approximately 38 percent protein or less, one third of which was from animal sources, appeared to be more prone to tumor induction. The principal vegetable protein used in trout formulas was cottonseed meal. Cottonseed meal is a variable product, depending on the area of production and the type of meal. It may be harvested early in the fall and not milled until late in the winter, so that changes in composition could result from storage. The principal sources of cottonseed meals for trout rations prepared in the Western States would be Arizona, California, and possibly Texas. Thus, in the studies it was necessary to use a single source of cottonseed meal. A single source is meant to be a single supplier, although the cottonseed meal could have originated from a variety of areas.

Fish meal, one of the most important components of trout diets, is prepared from different species of fish by a series of different methods. The most commonly used fish meals are prepared from menhadin, sardine, anchovy, herring, and cod and haddock byproducts. Neither sardine meal nor menhadin meal is commonly used in fish feeds. Herring meal is the principal fish meal used on the west coast for the production of trout feeds. White fish meal is used to a limited extent by producers of fish feeds located in the Midwest and Eastern States. In some cases the solubles, resulting from the concentrating of press liquor which is separated from the oil, are added back to the meal, resulting in a more complete meal.

White fish meal contains about 63 percent protein, 4.5 percent fat, 0.5 percent fiber, and 21 percent ash. It is processed by drying the waste products resulting from the filleting of cod and haddock. Its protein quality is excellent. Herring meal, on the other hand, is prepared from whole fish. The fish is precooked and the oil removed by pressing.

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The final product has a protein content of approximately 70–72 percent, fiber 1 percent, and ash about 11 percent. This is a high-grade product and is used principally by manufacturers of feeds located in the West. The South American fish meals are prepared from anchovies and other species of fish. These meals are generally between 65 and 68 percent protein.

The quality of fish meals varies quite widely, even from a given production area. This variation probably results from the methods of handling the fish prior to processing it into meal. If the fish when it is landed is of very fresh quality, the fish meal produced from this will be light in color and will produce a high quality feed. However, if the fish used to prepare the meal has begun to spoil, the oil will become rancid more rapidly, and the meal itself will be darker in color and generally of poorer quality. Thus, it is obvious that fish meals from different plants and different areas will vary in quality, and this quality could have an effect upon the animal consuming the feed. Thus, it was necessary in the planning of the experiments to include fish meals from different sources.

Tumors were present in trout fed feeds produced both in the eastern part and the western part of the United States. The feeds on the eastern seaboard would most likely have included white fish meal, although there was a chance that South American fish meal may have been available. In the West, the fish meal is usually B.C. herring, although South American and South African fish meals have been used to a limited extent. It appeared that fish meal, although a possible contributor to the tumor, was not a major factor, since widely different types of meals were incorporated into feeds which induced tumor.

It was necessary in the design of the experimental program to choose one principal source of fish meal. Since B. C. herring meal was the predominant meal available, this was chosen as the basic ingredient for the experimental feeds. A second fish meal was chosen for the experiments. This fish meal was from a

TABLE 5.—*Summary of results, 25–26 months, tumor incidence*

Hatchery	Food type	Percent cottonseed meal	Tumor		
			Male	Female	Combined
American Falls	{High protein / High fish meal}	12.5	20	10	14
Mackay	{Low protein / High fish meal}	7.8	0	18	10
Hagerman	{High protein / Low fish meal}	30.5	75	67	72
Glenwood	{Low protein / Low fish meal}	21.0	39	29	35
Yakima	{High protein / High fish meal}	0	0	0	0
Springville	{Low protein / Low fish meal}	21.0	39	29	35
Spokane	{High protein / High fish meal}	0	0	0	0
Lewiston	{High protein / Low fish meal}	31.0	64	83	70
Vancouver	{High protein / High fish meal}	0	0	0	0
Midway	{Low protein / Low fish meal}	31.0	50	58	55
Parkview	High fiber				

TABLE 6.—*Calculation of dose rate on the basis of food allowance*
[Assumption: A toxic agent present at a concentration of 0.1 percent in the source]

Time on feed	Food allowance g/kg/day	Source of toxic agent (percent of total food) 20	10	5 (Toxic agent g/kg)
0–1 month	120	7.2	3.6	1.8
1–2 months	100	6.0	3.0	1.5
2–4 months	80	9.6	4.8	2.4
4–6 months	60	7.2	3.6	1.8
6–8 months	40	4.8	2.4	1.2
8–10 months	20	2.4	1.2	0.6

South American source, and its history was not completely known, but the use of this meal provided a useful comparison.

"Distillers' solubles" is a very general term describing the dried or evaporated solubles prepared from fermented grains, such as wheat, rye, and sorghum. Some of the grains are used in the production of alcohol and are truly distillers' products, while others described as distillers' solubles are in reality fermentation solubles. The fermentation solubles result from the lactic acid fermentation of grains in the presence of SO_2. The liquid produced from this fermentation is a rich source of B vitamins from the meals and from the organisms responsible for the lactic acid fermentation. Similarly, the distillers' solubles are a rich source of B vitamins from the yeast-based fermentation. The extractives from the grains or solubles produced during the fermentation may have been a source of toxic substances; hence it was necessary to choose two different sources of solubles and to compare these as possible sources of agents which could cause tumor in the trout.

Wheat middlings, the other major variable in trout feeds, is rather consistent. This is a product of the milling of grains and is added as a source of starch and fiber. Wheat middlings will reflect the different sources of wheat and the cleaning or disinfestation processes used in the preservation of the dry wheat. The product represents only the milled grain and, since it is a process and the product results from the sieving of the milled grain product, any agent present in the product which could induce a tumor would most likely be as a result of chemical additives used to treat the grain or as a result of spoilage. The product selected would vary in relation to the area of wheat culture and the type of grain being milled and thus two sources of wheat middlings were considered in planning the experiments.

SUMMARY OF RESULTS

Results from the field trials (tables 4 and 5) lead to the conclusion that the most critical ingredient in the source of a hepatotoxic agent in pelleted feeds was cottonseed meal. Two types of fish meals and wheat middlings failed to evoke a response when cottonseed meal was not included in the formulation. The dose rate for a toxic agent can be estimated on the basis of food allowance (table 6).

EVALUATION OF COTTONSEED PRESS OIL AT THE STATE OF UTAH, LOGAN EXPERIMENTAL FISH HATCHERY

The preliminary survey of the incidence of hepatoma and the screening experiments carried out in collaboration with various state fish hatcheries all confirmed that the most critical factor in the disease was cottonseed meal.

As a consequence of this early information, a followup experiment was developed in which one source of cottonseed was processed into meal and oil. The pre-press oil was incorporated into experimental diets fed to trout from a single source at the experimental hatchery at Logan, Utah.

In the test, cottonseed oil from the known source was substituted for stabilized animal fat in a formula similar to those used in the earlier screening studies.

The rainbow trout (*Salmo gairdnerii*) eggs for the experiment were obtained from Trout Lodge, Soap Lake in Washington and after hatching they were divided into duplicate groups and fed the test and control formulas from the time of swim-up. The size of the eggs and the initial pick-off record are shown in table 7.

TABLE 7.—*Cottonseed experiments*
[Eggs from Trout Lodge, Soap Lake in Washington]

Date received	February 11, 1963
Date hatched	February 18, 19, 20, 1963
Egg size	257 per ounce
Hatched	97.1 percent
Cripples	2.81 percent

The food was prepared, using the facilities of the Logan Experimental Fish Hatchery. The feed formula and the vitamin supplement are shown in tables 8 and 9. All of the ingredients were obtained from commercial sources with the exception of the cottonseed oil and stabilized animal fat. The stabilized animal fat was supplied by a meat processing plant in Seattle, and the cottonseed oil was the pre-press oil fraction produced from a cottonseed provided especially for the College of Fisheries.

The experimental plan for handling the trout and for feeding was the same as originally set forth in the earlier hepatoma research program. The trout were examined at intervals and reduced in numbers to keep pond loading capacity within the specified limits. At periodic intervals, autopsies were performed and the lengths, weights, and liver conditions were recorded. Whenever a question existed as to the condition of the liver, histological specimens were prepared and interpreted in the manner outlined in the hepatoma experimental procedure.

Glycogen was determined histologically, using paraffin sections prepared from material fixed in alcohol-formalin and stained with the PAS procedure. The glycogen was determined on fresh material, using the method of Montgomery (1957). This latter method de-

TABLE 8.—*Cottonseed experiment—Logan Fish Hatchery*
Feed formula

	Control	Test
	%	%
Fish meal (B.C. herring or Peruvian) Protein—minimum 70 percent Fat—maximum 8 percent	35	35
Dried skim milk (spray dried) Protein—minimum 35 percent	5	5
Delactosed whey or N.M.C. whey product (spray dried) Protein—minimum 17.7 percent	10	10
Liver meal (unextracted—without glandular products) Protein—65–70 percent Fat—10–15 percent	4	4
Animal fat (stabilized)	2	
Cottonsed oil		2
Brewer's dried yeast (debittered) Protein—minimum 45 percent	5	5
Alfalfa meal (not fortified with fat or dust depressants)	2	2
Vitamin premix (see table 9)	0.4	0.4
Wheat middlings Protein—minimum 15 percent Fiber—maximum 6 percent	31	31
Salt	6	6

TABLE 9.—*Vitamin premix*
[For 100 pounds of finished feed]

Vitamin A	100 USP units
Vitamin D_3	18 IU
Vitamin E	3000 IU
Riboflavin	4500 mg
D-Calcium pantothenate	2500 mg
Niacin	5000 mg
Choline chloride	12,500 mg
Vitamin B_{12}	1 mg
D Biotin	20 mg
Ascorbic acid	20,000 mg
Thiamine hydrochloride	4500 mg
Pyridoxine hydrochloride	1000 mg
Folic acid	100 mg

Carrier to give a total weight of 0.18 pound.

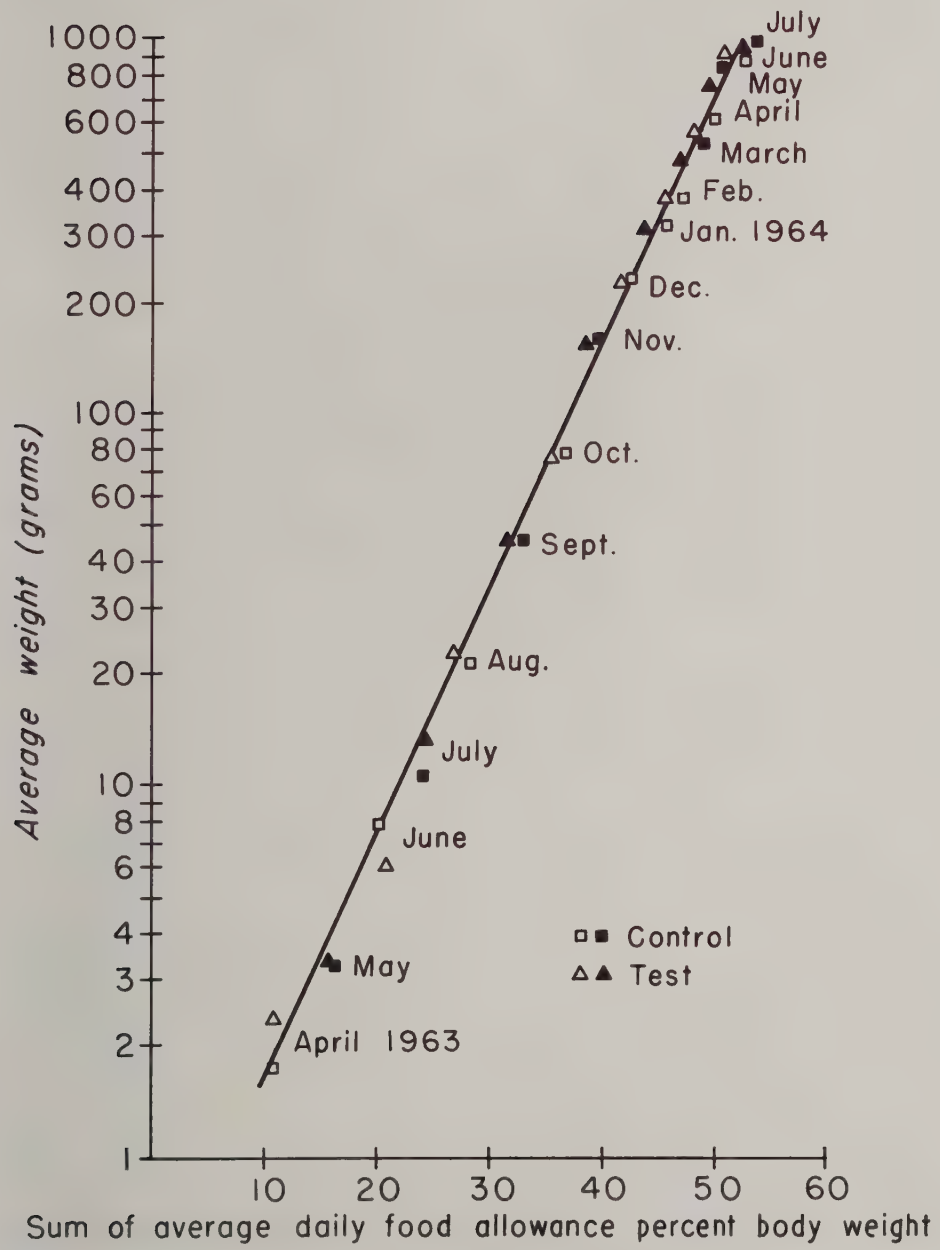

FIGURE 1.—Cottonseed oil experiment. Growth of rainbow trout (McCleary strain), State of Utah Experimental Fish Hatchery, Logan, Utah.

TABLE 10.—*Cottonseed oil test: Control group*
[Two values for each sampling period refer to replicates]

Month	Size at end of month g	Cumulative mortality %	Food per gain	Monthly gain %	Daily food allowance %
1963					
March	0.36 0.36	10.95 9.25	1.50 1.56	89.5 100.0	5.3–6.0 5.3–6.0
April	1.82 1.65	12.2 10.15	1.83 1.75	210 245	6.0–4.7 6.0–4.5
May	3.14 3.51	12.4 10.55	1.40 1.72	104 130	5.9–4.4 5.6–4.1
June	7.60 6.98	12.9 10.8	1.41 2.04	142 99	4.4–2.9 5.0–4.5
July	10.91 10.46	13.2 11.7	2.44 3.49	43.5 49.9	3.6–2.9 5.1–4.3
August	22.4 21.0	13.7 12.3	1.35 1.92	105 101	3.6–2.3 6.0–3.3
September	45.0 45.5	13.9 12.7	1.76 1.59	101 116	5.1–3.9 6.8–3.7
October	81.6 75.0	14.7 13.5	1.25 1.4	81.5 71	4.5–3.7 4.5–3.3
November	156.1 152.0	14.7 13.5	1.18 1.11	91 102	3.7–1.95 3.3–1.9
December	228.6 228.0	16.0 13.5	2.0 1.71	46.3 50.0	4.1–2.0 4.0–2.0
1964					
January	302.3 319.4	17.4 14.2	2.3 1.8	32.2 40.0	3.0–1.8 3.1–1.7
February	364.3 394.4	17.4 14.2	2.3 2.4	21.2 23.6	2.1 1.9 2.1 1.7
March	499.3 562.4	19.5 15.6	--- ---	37.0 42.6	1.5–1.1 1.6–1.2
April	597.6 606.4	19.5 15.6	--- ---	19.7 22.0	1.1–1.2 1.2–1.4
May	755 909	19.5 17.6	--- ---	26.3 50.0	1.2–1.4 1.2–1.5
June	899 909	19.5 17.6	--- ---	19.0 0	1.4 1.3 1.5 1.3
July	941 1025	21.5 17.6	--- ---	3.5 12.8	1.4–1.3 1.4–1.3

TABLE 11.—*Cottonseed oil test: Test group*
[Two values for each sampling period refer to replicates]

Month	Size at end of month g	Cumulative mortality %	Food per gain	Monthly gain %	Daily food allowance %
1963					
March	0.49 0.45	5.65 4.10	0.97 1.12	176 169	5.3–6.0 5.3–6.0
April	2.24 2.45	6.25 4.90	1.88 1.66	213 232	6.0–4.6 6.0–4.2
May	3.31 3.42	6.85 5.2	1.37 1.43	111 96.5	5.6–4.8 5.5–5.0
June	7.44 4.84	7.15 5.7	1.68 1.36	108 140	4.8–3.7 5.0–3.4
July	13.31 12.85	7.55 6.2	1.84 2.08	79 65.6	4.2–3.0 4.0–3.0
August	23.4 21.2	8.2 6.8	1.41 1.70	76.9 64.8	3.4–2.1 3.4–2.3
September	46.2 45.3	8.7 7.1	1.60 1.51	97.4 113.5	5.1–3.9 5.3–3.7
October	74.5 77.4	9.0 7.8	1.76 1.40	61 75	4.6–3.4 4.4–3.9
November	152.0 152.0	9.7 7.8	1.16 1.16	104 97	3.4–2.0 3.9–2.0
December	228.0 228.0	11.1 8.5	1.8 1.8	50 50	4.0–2.0 4.0–1.9
1964					
January	308.5 297.1	12.6 9.9	2.2 2.3	35.4 30.3	3.1–1.4 3.0–1.8
February	390.6 364.7	13.4 10.6	2.3 2.6	28.2 22.8	2.1–1.7 2.1–1.8
March	500.0 452.7	14.2 12.0	--- ---	28.0 24.8	1.6–1.3 1.6–1.4
April	598.5 529.7	16.2 16.0	--- ---	19.7 17.0	1.2–1.2 1.4–1.2
May	755.0 755.0	16.2 16.0	--- ---	26.2 42.6	1.2–1.3 1.2–1.3
June	909.0 909.0	16.2 20.0	--- ---	20.4 20.4	1.3–1.3 1.3–1.4
July	920.0 920.0	16.2 24.0	--- ---	2.0 1.2	1.4–1.3 1.4–1.3

pends on precipitating the glycogen with alcohol from the liver sample with KOH. The glycogen concentration was estimated colorimetrically, using phenol and sulfuric acid.

Results and conclusions

The growth of the trout, food conversion, and mortality were calculated from the data provided in the monthly hatchery reports (tables 10 and 11). The rate of growth, calculated on the basis of the average size of fish, was plotted vs. the cumulative daily food allowance calculated at monthly intervals (fig. 1). It is obvious on inspection of the data that the growth is directly related to the food allowed throughout the experimental period.

Samples were examined on April 9, 1964, and the results are shown in tables 12 and 13 and summarized in table 14. The glycogen in the livers of fish fed the control diet was about 1 percent, which was slightly higher than was found with the test diet (0.7 percent). The males tended to have slightly less liver glycogen than the females.

The ratio of liver weight to body weight was about 1 percent. As of this sampling date, all of the trout were immature, which may account for the smaller livers.

The fish fed the control diet all had normal livers. However, approximately 60 percent of the livers of the fish in the test group showed liver change on gross examination. In no case had these changes progressed to a tumor stage.

At the next sampling date, August

TABLE 12.—*Cottonseed experiment—Logan Fish Hatchery*
Analysis of samples taken 4-9-64

Sample No.	Weight g	Length mm	Condition index*	Liver				Mesentery fat
				Weight g	% Body weight	Glycogen %	Gross classification	
Control Diet								
Males								
6664	200	259	1.15	2.11	1.06		B	2
6666 [a]	447	330	1.24	3.94	0.88	1.60	B	3
6668	625	368	1.25	8.13	1.30	0.65	B	3
6670	578	351	1.34	4.30	0.74	1.13	B	3
6671	532	343	1.32	5.33	1.00	1.25	B	4
6694	390	325	1.14	3.06	0.78	0.08	B	3
6697	454	330	1.26	3.91	0.86	1.12	B	3
Average	461	329	1.24	4.40	0.95	0.97		
Females								
6665 [a]	403	318	1.26	3.91	0.97	0.16	B	3
6667	420	310	1.41	4.27	1.01	0.30	B	3
6672	468	333	1.26	3.77	0.81	0.62	B	3
6673	467	330	1.30	4.61	0.99	0.14	B	3
6669	385	318	1.20	3.33	0.87	0.89	B	3
6695	497	346	1.20	3.77	0.76	1.00	B	3
6696	512	340	1.30	5.95	1.16	1.56	B	3
6698	380	308	1.30	3.58		0.76	B	3
6699	532	335	1.41	5.45	1.01	1.63	B	3
6700	450	320	1.37	5.64	1.25	2.39	B	4
6701	484	358	1.05	4.53	0.94	0.97	B	3
6702	428	318	1.33	5.62	1.31	1.56	B	3
6703	603	356	1.33	5.21	0.86	1.63	B	3
Average	464	330	1.29	4.60	1.00	1.05		

* $c=\frac{W\times 100}{L^3(cm)}$

[a] Kidney granuloma on gross examination.

TABLE 13.—*Cottonseed experiment—Logan Fish Hatchery*
Analysis of samples taken 4-9-64

Sample No.	Weight g	Length mm	Condition index*	Liver				Mesentery fat
				Weight g	% Body weight	Glycogen %	Gross classification	
Test Diet								
Males								
6674	557	343	1.39	5.84	1.07	0.35	C	3
6676	475	346	1.15	3.65	0.97	0.58	C	3
6677	440	343	1.09	3.44	0.78	0.27	B	3
6692	423	330	1.18	3.12	0.74	0.66	B	2
6679	478	336	1.26	4.78	1.00	1.03	C	3
6681	562	356	1.25	3.72	0.66	0.14	C	3
6684	198	252	1.24	1.45	0.73	0.88	B	2
6685	360	310	1.20	2.99	0.83	1.34	C	3
6686	424	343	1.05	3.12	0.74	0.17	B	2
6687	408	328	1.15	3.09	0.76	1.00	C	2
Average	432	329	1.20	3.52	0.83	0.64		
Females								
6675 [a]	466	343	1.16	4.35	0.93	0.35	C	3
6678	213	262	1.18	2.44	1.14	0.24	B	3
6680	534	346	1.29	4.86	0.91			3
6682	270	279	1.24	1.82	0.68	0.26	C	2
6683	341	310	1.14	3.58	1.05	1.01	B	4
6688	397	325	1.16	3.42	0.86	1.50	C	3
6689	412	310	1.38	3.62	0.88	0.94	B	2
6690	512	336	1.35	5.00	0.98	1.08	C	3
6691	438	325	1.28	3.76	0.86	0.54	C	3
6693	526	340	1.34	5.80	1.10	1.04	C	3
Average	411	318	1.25	3.86	0.94	0.77		

* $c=\frac{W\times 100}{L^3(cm)}$

[a] Kidney granuloma on gross examination.

TABLE 14.—*Cottonseed experiment—Logan Fish Hatchery*
Samples taken 4–9–64
(Averages)

	Weight g	Length mm	Condition index*	Liver		
				Weight g	% Body weight	Glycogen %
Test diet						
Males	432	329	1.20	3.52	0.83	0.64
Females	411	318	1.25	3.86	0.94	0.77
Control diet						
Males	461	329	1.24	4.40	0.95	0.97
Females	464	330	1.29	4.60	1.00	1.05

* $c=\frac{W\times 100}{L^3(cm)}$

24, 1964, the same percentage of fish were found to have gross changes in liver condition as found in the earlier samples. The results of the examination are shown in tables 15 and 16 and summarized in table 17. It was quite evident that cottonseed press oil caused an abnormal response in the livers of trout and the frequency of this response was the same in both males and females.

The differences in cumulative mortality between the two groups merely reflects the first month's mortality, *i.e.* an average of about 5 percent as opposed to an average of about 10 percent. This higher mortality resulted from mechanical difficulties in handling the feed and should not be considered as an abnormal situation.

It is useful to note that in these studies two formulas were compared in

TABLE 15.—*Cottonseed experiment—Logan Experimental Fish Hatchery*
Analysis of samples taken 8–24–64
Control diet

Sample No.	Weight g	Length mm	Condition index*	Liver			Mesentery fat
				Weight g	% Body weight	Gross classification	
Male							
6774	940	400	1.45	8.67	0.92	B	4
6776	911	400	1.42	10.63	1.16	B	3
6778	1030	425	1.34	12.83	1.25	B	4
6781	857	403	1.30	10.55	1.23	B	4
6784	990	420	1.34	11.03	1.11	C	4
6786	852	390	1.44	8.45	0.99	B	4
6799 [a]	1028	420	1.39	9.10	0.89	B	3
6801	890	406	1.33	9.47	1.06	B	3
6802	980	430	1.22	11.45	1.17	B	3
Average	942	410	1.36	10.24	1.09		
Female							
6775	1161	435	1.41	12.09	1.13	C	4
6777	745	388	1.28	6.80	0.91	B	3
6779	1215	432	1.50	13.58	1.12	B	3
6780	647	360	1.39	6.86	1.06	B	4
6782	825	410	1.20	9.52	1.15	B	4
6783	660	374	1.27	6.58	1.00	B	4
6785	1030	426	1.32	11.10	1.08	B	4
6787	915	415	1.27	8.01	0.88	B	4
6788	1052	435	1.28	14.43	1.37	B	4
6800	890	397	1.42	11.55	1.30	B	3
6803	825	397	1.31	14.75	1.79	B	3
Average	906	406	1.33	10.48	1.16		

* $c=\frac{W\times 100}{L^3(cm)}$

[a] Kidney granuloma on gross examination.

TABLE 16.—*Cottonseed experiment—Logan Experimental Fish Hatchery*
Analysis of samples taken 8–24–64
Test diet

Sample No.	Weight g	Length mm	Condition index*	Liver			Mesentery fat
				Weight g	% Body weight	Gross classification	
Male							
6766	1065	468	1.04	10.74	0.92	C	3
6768	670	380	1.22	6.82	1.02	B	3
6769	1050	400	1.64	10.46	1.00	C	3
6806	765	385	1.34	7.29	0.95	B	3
6809	925	420	1.25	9.83	1.06	C	2
Average	895	411	1.30	9.03	0.99		
Female							
6764	1130	430	1.42	12.36	1.09	C	4
6765	629	358	1.37	6.93	1.10	B	3
6767	888	400	1.38	8.09	0.91	B	3
6770	995	427	1.27	9.66	0.97	C	3
6771	810	375	1.53	7.52	0.95	C	3
6772	689	370	1.35	7.60	1.10	B	3
6773	825	406	1.22	8.78	1.06	C	3
6804	954	410	1.38	11.64	1.22	C	4
6805	980	418	1.33	15.00	1.53	B	4
6807	695	400	1.08	6.99	1.00	B	4
6808	880	395	1.42	9.06	1.03	B	4
6810	1232	448	1.37	14.00	1.14	C	3
6811	985	435	1.20	10.80	1.10	C	3
6812	893	435	1.09	8.38	0.94	C	3
6813	895	410	1.30	10.13	1.13	C	4
Average	899	408	1.32	9.80	1.08		

* $c=\frac{W\times100}{L^3(cm)}$

which the type of fat was varied. The results suggest that when a higher melting point fat is added to a standard trout ration, there is no significant effect on growth, as compared with cottonseed oil, a vegetable oil source. There are obvious differences in the histology of the livers, in that fat deposits were minimal and could not be considered pathological under this condition of feeding. In the overall picture, this type of diet supported very good growth and survival. Small abnormalities were noted in the livers in the case of the animal fat formula, but these abnormalities could not be considered pathological. The pre-press oil from cottonseed proved undesirable for incorporation in trout feeds. However, other sources of cottonseed oil may be satisfactory and should be tested.

REFERENCE

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TABLE 17.—*Cottonseed experiment—Logan Fish Hatchery*
Samples taken 8–24–64
(Averages)

	Weight g	Length mm	Condition index*	Liver	
				Weight g	% Body weight
Test diet					
Males	895	411	1.30	9.03	0.99
Females	899	408	1.32	9.80	1.08
Control diet					
Males	942	410	1.36	10.24	1.09
Females	906	406	1.33	10.48	1.16

* $c=\frac{W\times100}{L^3(cm)}$

DESCRIPTIVE CLASSIFICATION ON NORMAL AND ALTERED HISTOLOGY OF TROUT LIVERS

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Abstract.—Wax-plate reconstruction of non-neoplastic trout liver has revealed an architecture consisting of cell plates and true hepatic cords. The former predominate and are 2 cells in thickness. Thickness of cell plates alone is reported to be an unreliable criterion of hyperplasia in histological preparations since the plane of sectioning may often bisect the two rows of cells within cell plates. Diameters of nuclei in normal liver cells have been found to increase as body weight increases. Evidence is presented to suggest direct continuity between hepatocytes and cells of the terminal biliary system. Active hematopoiesis is reported to be a typical feature of the normal liver. Tumor prevalence approaching 100 percent is found to be attained in 1 year among groups fed a "positive control" diet. Detailed constitution of this diet is provided in addition to complete formulation of diets which did not produce hepatic alterations in 24 months of continuous administration. Feeding of these diets has provided tumorous and nontumorous hatchery fish which are compared with wild trout. A classification is proposed to aid in providing a descriptive record of liver alterations.

Several papers in the current series contain photographs depicting histological features of normal and tumorous trout livers. These are not to be repeated here. The objectives of the present study are to provide a more detailed description of the normal liver and to suggest a classification scheme to simplify identification of departures from the normal condition.

Livers of more than 1,200 hatchery fish have been examined in approach to the objectives. Methods of staining and fixation are described in other papers of this series.

More expansive treatment of the proposed classification is to be formally published.

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METHODS AND MATERIALS

Fish which form the basis of this report were sampled quarterly during a 2-year period which commenced with newly hatched fish. Ten to twenty fish were obtained from each group at each sampling. These were taken at random from a large number captured by dip net after attracting with food. Trout were kept alive in large buckets and killed individually for examination. In all, 1,200 samples were obtained from three hatcheries.

One hundred and fifty wild rainbow trout were collected from Cabin and Corral Creeks, in Challis National For-

est, Custer County, Idaho. Hatchery trout had not been released in these streams for 15 years.

Routine autopsy data were collected for each fish coincident with fixation of tissues for histological examination. These data include sex, age, length, weight, amount of visceral fat, presence of parasites, liver weight, and appearance of the liver. An arbitrary designation of gross liver appearance was assigned as follows:

A—uniform red-brown coloration, no nodules evident.

B—non-uniform coloration and/or suspected enlargement, no nodules evident.

C—one or more nodules, less than 3 mm diameter.

D—one or more nodules, 3 mm to 10 mm.

E—one or more nodules greater than 10 mm.

HATCHERY REGIMEN

Embryonated "eggs" at an advanced stage of development (eye pigments visible) were distributed to three trout hatcheries. These stations were the Idaho Department of Fish and Game trout hatchery near Mackay, Idaho, the Washington Game Department trout hatchery near Vancouver, Wash., and the Washington Game Department trout hatchery at Yakima, Wash. Three groups were replicated at each hatchery and were reared in separate concrete ponds.

At no time did these fish receive nutrients other than the diets defined in tables 18, 19, and 20 with the exception that a minute contribution may have been made by natural food from the water supplies. The "positive control" diet was the same as the positive control employed by Wolf and Jackson [*15*][1] which was known to effect selective tumor response in the trout liver. Diets III, XII, and XIV were original formulations for purposes of the present study. All diets were compounded by a commercial feed manufacturer according to the specifications in tables 19 and 20. Diets were fed in amounts which varied according to size of fish and water temperature as described by Leitritz [*6*]. Instructions of this author

[1] Numbers in brackets refer to references at the end of this paper.

TABLE 18.—*Test groups of trout at three hatcheries*

Hatchery and diets	Source of eyed eggs	Date of first feeding	Number surviving at first feeding
		1961	
Idaho State Game Department Hatchery, Mackay, Idaho:			
Positive control diet	Shasta, Calif.	Mar. 17	28,600
Do	Soap Lake, Wash.	Mar. 13	25,550
Diet III	------ do ------	Mar. 13	104,500
Washington State Game Department Hatchery, Vancouver, Wash.:			
Positive control diet	Shasta, Calif.	Mar. 14	16,000
Do	Ford, Wash.	Mar. 20	16,500
Diet XII	------ do ------	Mar. 20	33,000
Washington State Game Department Hatchery, Yakima, Wash.:			
Positive control diet	Shasta, Calif.	Mar. 18	17,300
Do	Ford, Wash.	Mar. 18	14,400
Diet XIV	------ do ------	Mar. 18	28,800

TABLE 19.—*Experimental trout food formulas*
[Ingredients = parts per hundred]

Ingredient	Positive control (parallel feeding at each hatchery)	III Mackay	XIII Vancouver	XIV Yakima
Fish meal–B.C. herring	19.0	26.5	34.6	31.6
Meat meal	7.0	6.5	9.0	8.4
Brewer's yeast	6.5	6.5	9.0	8.4
Dried skimmed milk	3.4	3.3	4.7	4.2
Cottonseed meal	18.0	8.6	------	------
Distillers' solubles	7.0	6.5	9.0	17.1
Salt (iodized)	2.4	1.1	1.6	1.4
Vitamin premix A (see table III)	------	0.35	0.35	0.35
Vitamin premix B (see table III)	0.2	------	------	------
Vitamin A & D feeding oil (Vit. A 2250, Vit. D 300)	2.0	2.2	3.1	2.8
Blood meal	2.5	------	------	------
Wheat middlings	24.0	16.5	14.4	13.2
Alfalfa	0.6	------	------	------
Kelp meal	------	3.3	4.7	4.2
Liver meal	6.7	6.6	9.4	8.4
Beet pulp	------	11.9	------	------

TABLE 20.—*Vitamins supplied by premixes*
[To supply vitamins g/100 lbs. of feed]

Vitamin	Grams of vitamin per 100 pounds of feed supplied by—	
	Control (premix B)	Diets I—XIV (premix A)
Ascorbic acid	5.0	54
Biotin	0.02	0.012
Calcium pantothenate	1.5	4.8
Choline·HCl	30	27
Folic acid	0.08	0.18
Inositol		60
Menadione		0.21
Niacin	4.5	4.8
Para-aminobenzoic acid	0.8	
Pyridoxine·HCl	0.3	0.6
Thiamine·HCl	0.3	1.5
Alpha-tocopherol		2.4
Riboflavin	0.9	1.2
B_{12} concentrate	0.0008	0.0002
E supplement	0.21 IU	
A	1.0 IU	
D_3	50,000 IU	
dl-methionine	9.1	

were also followed in the periodic reductions in number of fish which were necessitated by growth.

LIVER REGENERATION

Partial hepatectomies were performed on four trout from the Vancouver Hatchery to gain some information on regenerative activity in the trout liver. These fish were killed 1 week and 2 weeks following removal of the posterior one quarter inch of the liver. Surgical techniques originated by Robertson [*11*] were employed. All fish were without tumors and had been fed diet XIII for 12 months.

STRUCTURE OF THE NORMAL LIVER

A wax-plate reconstruction was prepared using 15 serial sections of a portion of the liver of a normal, wild trout. Each section was photographed with reference to a particular central vein. The model was prepared at a scale of 1:1000 by tracing outlines of sinusoids on sheets of dental wax. Vascular spaces and cell plates were reconstructed in separate models.

NUCLEAR MEASUREMENTS

A further attempt to quantify the structure of the normal liver was made in a series of 1500 measurements of liver cell nuclei. Trout from four areas were included (table 21). Measurements were made at a magnification of 2,500 to the nearest half-micron with a calibrated ocular micrometer. Each nucleus was measured in one plane only; that which was lateral in the field of observation. Despite the fact that some nuclei are elliptical, the mean of a series of measurements performed in this manner provides an adequate estimate of the mean diameter obtained from multiple measurements of individual nuclei. No attempt was made to select nuclei. Scanning was begun at the right edge near the center of each section. The initial point of origin was chosen out of focus. Nuclei were not measured if (*a*) they did not intercept the superimposed scale as the slide was moved laterally, and (*b*) if the nuclear membrane was not intact. Additional measurements were made of nuclei from selected areas in abnormal livers for purposes of comparison.

CLASS I—THE NORMAL LIVER

The color of the liver in freshly killed trout ranges from light to dark red brown. Occasional livers which are very rich in glycogen may assume a mottled appearance with cream-colored blotches or streaks on the liver surface. The

TABLE 21.—*Measurement data, liver cell nuclei of nontumorous fish*

Hatchery or locality	Age of fish (years)	Number of fish in sample	Measurements per fish	Total nuclear measurements
Vancouver Hatchery, Wash.	2	5 males 5 females	30	300
Vancouver Hatchery, Wash.	1	5 males 5 females	30	300
Yakima Hatchery, Wash.	2	5 males 5 females	30	300
Mackay Hatchery, Idaho	2	5 males 5 females	30	300
Cabin and Corral Creeks, Idaho	2–3	5 males 5 females	30	300
Total				1,500

liver is not usually divided into distinct lobes and ranges in shape from somewhat triangular to almost rectangular; the latter are often found in larger females presenting an apparent sex dimorphism. The anterior and largest portion of the organ lies in close proximity to the pericardial septum and is most frequently located almost entirely within the left half of the coelomic cavity. The livers of normal hatchery trout in the present study averaged 1.4 percent of the total body weight with sample means fluctuating between 1.1 percent and 1.7 percent.

Histologically, the liver lobules are extremely indistinct and only roughly defined by the positions of central veins and portal triads. Hepatic triads are represented by distinct bile ducts and portal veins, while the artery is often smaller than commonly encountered in mammalian livers. Fibrous tissue is scanty except in association with portal vessels. Bile ducts are frequently surrounded by lymphoid tissue with melanin variably interspersed among or within lymphocytes. The lymphoid tissue has been shown to have an active hematopoietic function since the administration of colchicine (5 mg/kg intraperitoneal injection with 5 days delay before excision) results in accumulation of many metaphases in component cells.

Wax reconstruction shows the plates of hepatic muralia are 2 cells thick. These two-layered sheets of cells are interconnected with similar sheets and are perforated by sinusoidal spaces which wind tortuously, branching, anastomosing, and rebranching in such a manner that any sinusoid is commonly connected to all other sinusoids.

The relationship of cell plates to sinusoids is like that of a mold to the casting material, as noted by Elias and Bengelsdorf [*2*] with whom the present visualization of architecture agrees very well. Contrary to the implication of these authors (op. cit.), however, it is not difficult to find rosettes of six to eight hepatic cells in the trout suggesting a minor component in interconnections of cordlike nature. Bands of eight or more cells in *width* are not uncommon where the plane of sectioning has bisected a sheet of 2 cells *thickness.* For this reason the appearance of occasional cell plates more than 2 cells in width has not been viewed as atypical unless accompanied by numerous mitoses and changes in cytoplasmic basophilia.

The most commonly encountered cell plates are 2 cells in thickness, bounded by a distinct endothelium and separated from other plates by sinusoidal spaces. Weak polarization of nuclei is often observed with nuclei tending to be displaced toward the sinusoids.

This finding differs from the interpretations reported by previous authors [*9, 17*].

In younger fish (age 2–6 months) the sinusoids appear to be distended. This feature is consistent and apparently normal in young trout.

Bile canaliculi appear to be arrayed in branching, weblike fashion between contiguous parenchymal cells. In OsO_4-fixed material the branches may be seen to diverge from a centrally-placed canaliculus and advance between cells to terminate short of the endothelium. The width of canaliculi is approximately 1 μ. The electron microscope has revealed that the bile canaliculus in mammals does not have a true cellular wall as formerly believed, but rather is bounded solely by cytoplasmic membranes of adjacent hepatocytes.

Small ducts bounded by two biliary cells occur with such frequency in the trout as to constitute a regular feature of the terminal biliary system. These ducts are disposed between the two

rows of hepatocytes which typically constitute the cell plates. When cord-like rosettes of parenchymal cells are viewed in cross section, the center is often marked by the nucleus of a biliary cell. More rarely, the opposed nuclei of two biliary cells are noted. Cytoplasmic intrusions have been observed from biliary cells into spaces between hepatocytes suggesting that canaliculi may be very short, or perhaps supplanted in some instances by processes of biliary cells.

CYTOPLASM OF NORMAL HEPATIC CELLS

Trout fed with standard levels [*6*] of dry diets typically display moderate cytoplasmic basophilia in hepatic cells. Not infrequently the parenchymal cells are highly vacuolated when examined in H and E paraffin sections. Fresh-frozen sections from the same liver, when stained by the PAS method (with complementary diastase controls) and with oil red-0, indicate that glycogen is responsible for the great bulk of this vacuolation. At high magnifications a general dispersion of fine fat droplets (1 μ and less) is demonstrated by oil red-0 staining.

The "fatty" vacuolated livers depicted in H and E preparations by Wood et al. [*16*] and by Nigrelli and Jakowska [*9*] bear a striking resemblance to glycogen vacuolation as demonstrated histochemically in the present study and in that of Scarpelli et al. [*13*]. Fatty vacuolation has been observed occasionally (about 1 percent) in livers of tumor-bearing trout but in normal livers does not seem to constitute a prominent feature of overall histology. It has further been determined that glycogen is absent and no vacuoles are present in the liver cells of hatchery trout following complete withdrawal of food for 2 weeks.

NUCLEI OF NORMAL HEPATIC CELLS

Parenchymal nuclei are round or spherical with nuclear membranes sharply defined by basic stains. A single, prominent nucleolus is most frequent and often approximates a central position. Fine strands or clumps of chromatin constitute a typical feature. The nuclear space otherwise appears vacant following routine fixations and staining.

Measurements of nuclei have proven to be informative. Nuclear diameters in non-neoplastic trout were found to vary with sex and body weight in a manner independent of age. Nuclear diameters increased as body weight increased.

The influence of sex on nuclear diameter was found to be significant at the 95 percent level ($F=5.18$ with 1,30 d.f.) with consistently larger nuclei found in males. These differences could not be attributed to differences in body weight since males most often weighed less than females (table 22).

TABLE 22.—*Diameters of liver cell nuclei in four trout populations, age 2 years*

Measurements	Sources			
	Mackay	Vancouver	Yakima	Wild
Males (5), mean of 150 measurements of nuclear diameters (μ)	5.40	6.66	5.77	5.38
Females (5), mean of 150 measurements of nuclear diameters (μ)	5.31	6.56	5.74	5.06
Male/female ratio	1.063	1.015	1.005	1.063
Males, mean body weight (g)	385.6	1954.8	521.8	57.6
Females, mean body weight (g)	447.4	2198.4	536.8	41.6

These data agree with the findings of Sauser [*12*] but reflect a lesser difference than Sauser reported ($\delta / \varphi = 1.09$).

Diameters of nuclei in 2-year-old fish from three hatcheries and two wild populations (table 22) were compared

by an analysis of covariance with fish weight and liver weight as covariates. Differences in nuclear diameter were found to be strongly significant between (but not within) the populations ($F=-5.3$ with 3,30 d.f.). Further data were collected on body weight and nuclear diameters of fish from these same localities to provide a reasonably continuous range in body weights. Since only one population contained fish larger than 800 g at 2 years (Vancouver Hatchery) all larger fish were obtained by necessity from that population. It was further necessary to reexamine liver slides from younger fish (18 months) from the Vancouver Hatchery to obtain body weights in the range of 800–1400 g for nuclear comparisons. A linear regression analysis of nuclear diameter vs. body weight indicated a highly significant positive slope of 0.00065 ($t=35.6$ with 48 d.f.). This relationship indicates an increase in mean nuclear diameter of 0.65 μ for each kilogram of increase in body weight. These data did not conform to the distribution of a polynomial of degree 2, and are thus evidently distributed in a linear fashion over the range measured.

A comparison of nuclear diameter and liver weight has indicated a similar trend of very much poorer linearity. This result might be expected since the liver size (as percent of body weight) may increase or decrease at different feeding levels, while body weight is less subject to fluctuation.

The factors responsible for increase in nuclear diameter with increase in body weight are not clear at present. It is suspected that genetic differences between populations do not play a part since fish at Yakima and Vancouver hatcheries were of the same "strain." Among factors which could contribute to the observed differences are the following possibilities: differences in water chemistry; differences in ploidy of hepatic cells; differences in hatchery effects (crowding, feeding, handling, etc.). Whatever these factors might be, it is evident that fish weight must be taken into account in any study concerned with the range of normal nuclear diameters. Nuclear diameters are doubtlessly of importance when attempting to describe the early changes from the normal range found in those fish fed the tumor-evoking diet (see class II below). The confidence limits of the distribution have been found to contain invariably the mean of 30 nuclear measurements per fish in non-neoplastic trout from other sources (including newly hatched fry).

REGENERATION FOLLOWING PARTIAL HEPATECTOMY

Little evidence of regeneration was noted 1 week postoperatively. After 14 days cellular components of the regenerating nodes included lymphocytes, red blood cells, fibrocytes and strands of proliferating hepatocytes. These strands were occasionally noted to possess a lumen, but more typically resembled solid cords. Regenerative cords typically displayed increased basophilia. Nodular regeneration was not observed during the brief period reported. The striking similarity of regeneration following partial hepatectomy to that accompanying dietary injury (class II) seems more than superficial. Further generalizations do not seem warranted due to sample limitations.

CLASS II—PRENEOPLASTIC DYSPLASIA WITH REGENERATION

Within 40 days after feeding began, average fish length and weight were approximately 1 g and 36 mm respec-

TABLE 23.—*Cellular features typical of dysplastic changes at 90 days*

	Basophilic cells	Pale cells
Cytoplasmic volume compared to normal	decreased	increased
Cytoplasmic basophilia	intense	nil
Mean nuclear diameter	5.8 μ	12.7 μ
Nuclear inclusions	absent	occasional
Nuclear invaginations	rare	common
Mitotic activity	pronounced	absent

tively. Livers were normal in gross appearance. Microscopical evidence of non-localized dysplasia was first noted at this time. Cell plates had become difficult to recognize as a consequence of marked enlargement of original parenchymal cells. Diameters of hepatocyte nuclei frequently exceeded 8 microns (mean of 100=8.2 μ) in distinct contrast to nuclear dimensions obtained from fish fed diets other than the positive control.

More than 70 percent of samples collected at 90 days displayed two prominent cellular components which differed from normal hepatocytes. The first is represented by continued fading and enlargement of original parenchymal cells (subsequently termed "pale cells"). The second cell type is similar morphologically to the normal liver cell but differs in possessing a lesser cytoplasmic volume and marked affinity for basic stains. Additional features are summarized in table 23.

CLASS IIIa—BASOPHILIC NODULES OF PARENCHYMALIKE CELLS

As suggested by the topic heading, the nodules IIIa consist predominantly of one cell type, that resembling the liver cell. Occasionally, *normal* biliary elements may be contained within these "early" nodules to include (*a*) encircled intrahepatic bile ducts, and (*b*) individual cells of the terminal biliary system. These elements of the normal biliary tree are to be later distinguished from the atypical, degenerative, pseudoducts which are a regular feature of class IIIb. Distinction from class IIIb seems to be meaningful both in the sense of progressive change, and in terms of gross and microscopic morphology.

Nonencapsulated nodules with enhanced basophilia were first noted after 5 months in groups fed the positive control diet, as previously noted. Neither at that time, nor subsequently, were proliferating nodules observed in groups fed the other experimental diets. In earliest gross appearance the nodules are whitish spots smaller than 1 mm diameter. Thickness of cell plates may range from 2 to more than 20 cells. These nodules are seemingly disposed at random. Similar spots may be caused by parasitic infestation, bacterial infection, inflammation, or the healed lesions of these processes, thus demanding microscopic confirmation.

Ashley and Halver [*1*] have reported that the "liver lipoid degeneration" found in European trout hatcheries [*3,4,5*] is the same as ceroid liver disease observed in U.S. trout culture. Although both occurrences may eventuate in frank malignancy, further analogy seems unwarranted. Findings of the present study on seven trout from France have revealed positive, parenchymal cell reaction to all of the following tests: Schmorl's reaction, Sudan Black b, Ziehl-Neelsen acid-fast stain, PAS reaction, and fluorescence under ultraviolet illumination. These reactions are typical of ceroid-type lipofuscins, as described by Pearse [*10*] and identical to the results of Ghittino [*4*] on hatchery trout in Italy. Ceroid has been reported in U.S. trout by Wood et al. [*16*]; however, only in localized concentrations, viz. near bile ducts. Massive intracellular accumulations of "ceroid" have never been observed during the

present study in trout livers other than those obtained from France.

CLASS IIIb—BASOPHILIC NODULES CONTAINING CHOLANGIOMATOUS ELEMENTS

Tumors which had attained a diameter of 5 mm or more were frequently found to have undergone degenerative changes in the central portion. These degenerative changes typify the present class and distinguish it from class IIIa, although both classes often coexist in the same fish, but at separate loci.

The earliest alteration (in the seventh month) was seen in the appearance of a sparse fibrous stroma near the center of larger tumors. Tumor cells which contact this fibrous tissue are seemingly transformed to atypical ductlike structures. It should be emphasized that these "pseudotubules" [*14*] are enclosed within the tumor and have never been observed to contain mitotic figures. Small islands of tumor cells appear to be entrapped by the advance of fibrous tissue which accompanies tumor growth. These trapped islands of cells range in appearance from typical tumor cells to small lumenized ductules, to enlarged, aberrant, glandlike structures. Transitional structures are sometimes evident.

The "pseudotubules" disappear centrally with continued advance of fibrous elements in such a manner that the tumor is comprised of an exclusively fibrous central zone, an intermediate zone containing tubules and transitional structures, and an outer zone of typical tumor cells.

A main feature in cells of smaller elements is their minimal cytoplasmic volume which serves to distinguish them from normal bile ducts. Larger glandlike structures are often composed of columnar cells but their erratic courses distinguish them at once from normal. The prevalence of these larger glandular alterations approximates 5 percent of specimens classed IIIb.

With continued enlargement of the tumor to 10 cm or more, a variety of gross changes are apparent which include conditions ranging from solid admixtures of tumor cells and fibrous tissue to a shell of tumor and fibrous cells bounding a globular cavity filled with blood-tinged fluid. The abdominal wall is often distended to an extreme degree. Good color photos are provided by Ghittino and Ceretto [*5*] and by Levaditi et al. [*8*], which reveal the variability in gross aspects.

These bulky tumors are frequently associated with adhesions to peritoneum, pyloric caeca, or digestive tract, but true invasiveness has not been demonstrated during this study.

CLASS IV—PROLIFERATING DUCTULES ASSOCIATED WITH ADVANCED TUMORS

Two microscopic features distinguish the class from others: (a) Ductules are abundant outside the tumor and (b) abundant mitoses are evident in ductule cells. On gross examination, a distinctive yellow mottling of the liver surface is noted. Areas of involvement are principally the posterior tip of the liver and the ventral free margin which apposes the abdominal wall. Affected sites are neither elevated nor depressed from the liver surface. Microscopic examination of yellow areas reveals necrosis, fibrosis, and ductular proliferation, with abundant mitoses in ductular cells. "Normal" hepatic tissue is interspersed in immediate contact with ductular structures, while no contact is apparent between ductules and hepatocellular tumors.

The lesion was first noted in fish 12 months old. Essentially 100 percent of fish fed the positive control diet are

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tumorous prior to the appearance of proliferating ductules. There is little doubt that this ductular proliferation is associated with deleterious dietary factors; however, the process might not be concerned with tumorigenesis proper. For this reason, no involvement toward malignancy is implied. The class is intended to note a secondary manifestation of dietary injury which follows the appearance of extensive tumorization.

Except for the extratumoral location and mitotic competence observed, these ductules are very similar microscopically to those of class IIIb. This similarity is particularly striking in the invariable association of ductular cells with fibrous tissue.

CLASS V—HEPATOCELLULAR CARCINOMA

There would appear to be little question that the advancing tumors described in previous classes are any different from those described by other authors. Failure to observe metastases during the present experimental period is believed to reflect the findings of other authors [*5, 7*] to the effect that hepatic tumors in trout are relatively late in metastasizing. Malignancy has been reported most often in fish aged 3 to 8 years.

Most authors concerned with trout hepatomas have described malignant lesions. A brief description is included here for the sake of completeness, which has been based on materials from six fish of different age and dietary history from those which form the bulk of this report.

A striking feature of malignant lesions is the presence of two or more populations of cells with different morphology. One common cell type in primary tumors might be judged to be well differentiated, however, these parenchymalike cells are to be found in metastases. Metastatic cells displaying different degrees of anaplasia are often established in close proximity. More anaplastic cells are often less basophilic. The limited samples of the current study have not revealed tumor giant cells, although such cells have been reported by Ashley and Halver [*1*].

Some of the malignant tumor cells display little anaplasia. The small, regular nuclei of these malignant cells cannot be said to differ from those typical of very early, small tumors. It would, therefore, appear that malignancy cannot be satisfactorily ascertained by appearance of tumor cells. Thus, in the absence of metastases, neither malignant nor benign designations are considered to be well founded where cell morphology is the sole criterion.

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Discussion

DR. SIMON: We have presumed that there is some value in presenting a classification of various morphological appearances of these liver alterations in trout fed dry diets, and to narrow this down we have specified particular diets, because it would appear that quite different things or additional things may appear on consequent feeding of other diets. So this is in the case of a particular diet in terms of descriptive morphology solely by light microscopy. This classification business, then is intended perhaps to describe features of the normal liver that have not been extensively described previously, and additionally to provide some means of escape from terminological difficulties, which I think are summarized fairly adequately in this report.

Another feature of the normal liver

has not been commented on very greatly, although I think numerous people here are familiar with it. There appears to be active hemophoiesis around at least the principal bile ducts, and there is some modest documentation of why this seems to be the case. But at any rate, there is a scattering of these cells around normal bile ducts.

An additional feature of interest is the fact that where the cytoplasm of biliary cells is noticeable there seem to be continuations that extend between and perhaps into hepatocytes, in other words, direct communication between the cellular portion of the biliary system and hepatocytes. This does not appear to be very common in mammals, where ordinarily the biocanaliculus is just formed by the borders of adjacent hepatocytes and tied together by desmosomes to make sort of a pseudocellular structure of the canaliculus.

Scarring in the center of the tumor progresses in such a way from the center outward with the expansion of the tumor such that there is a central zone largely of fibrous tissue, a transitional zone which contains ductlike structures, then an outer zone which is composed almost exclusively of typical tumor cells. This seems to be a sort of degenerative change that accompanies the deterioration at the center of these tumors.

HEPATOMA IN SALMONIDS: THE ROLE OF COTTONSEED PRODUCTS AND SPECIES DIFFERENCES

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The work described here may be divided into three parts:

1. Repetition of previous work demonstrating induction of hepatomas in rainbow trout by feeding cottonseed meal.

2. Feeding cottonseed flour to rainbow trout.

3. Feeding cottonseed meal to selected salmonids.

MATERIAL AND METHODS

1. Diets were based on the Santa Monica [1] ration as follows:

DA—Santa Monica (contains 20 percent cottonseed meal)

DB—Santa Monica—without the cottonseed meal portion

DC—Santa Monica—with 20 percent cottonseed flour substituted for the cottonseed meal

CO—Regular hatchery diet (cottonseed meal excluded in feed contract specifications, but particle analysis has indicated its variable presence).

2. Fish tested were rainbow trout (*Salmo gairdneri*), fall-spawning Shasta strain.

3. Management—The fish were fed and cared for in the standard hatchery manner except for their not being graded in size.

4. Facility—All fish were reared at the Darrah Springs Fishery. The water temperature there ranges from 58° to 60° F.

5. Sampling of the four diet groups of rainbow trout was carried out monthly. Twenty-five fish were taken from each group. Each fish was weighed, the liver examined, and coded specimens submitted for histologic examination to Dr. George Loquvam, Western Laboratories, Oakland, Calif.

PRELIMINARY RESULTS

Rainbow trout and cottonseed meal

Rainbow trout fed the Santa Monica diet (DA) showed hepatomas at 5 months of age, and by the ninth month an 88 percent prevalence was seen. The trout on the two control diets, DB and CO, have shown one microscopic hepatoma on the CO ration in the eighth month. No hepatomas have been seen in the DB samplings (see table 24).

TABLE 24.—*Hepatoma in rainbow trout by diet and age, Darrah Springs, Calif., 1964–65*

Age of fish (months)	*DA* 20% cottonseed meal	*DB* No cottonseed meal	*DC* 20% cottonseed flour	*CO* Regular diet
1	0/24*	0/25	0/25	0/25
2	0/25	0/25	0/25	0/25
3	0/25	0/25	0/25	0/25
4	0/25	0/25	0/25	0/25
5	7/25	0/25	0/25	0/25
6	12/25	0/25	0/25	0/25
7	19/25	0/25	0/25	0/25
8	19/25	0/25	0/25	1/25
9	22/25	0/25	5/25	0/25
10	n.d.	0/25	5/25	n.d.

* Number with tumors/number examined.

TABLE 25.—*Aflatoxin and gossypol content of cottonseed ingredients*

Cottonseed flour / Dietary ingredient	Aflatoxin B_1* (ppb)	Percent total* gossypol	Percent free* gossypol
	0	0.99	0.046
Cottonseed meal	36	0.71	0.40 †

* Analyses done by R. O. Sinnhuber, Oregon State University, Corvallis, Oregon.
† Subsequent review of this result indicates the value is 0.04.

Rainbow trout and cottonseed flour

In the samplings, no significant differences in the average weights have been observed between the four diet groups of rainbow trout (DA, DB, DC, and CO).

No hepatomas were observed in the cottonseed flour group (DC) until the ninth month, when 5 out of 25 were positive (table 24). In contrast, rainbow trout fed 20 percent cottonseed meal (DA), had hepatomas at 5 months of age, and by the ninth month 22 out of 25 sampled had hepatomas.

Biochemical studies indicated in tables 25 and 26 show the agents considered in attempting to account for the more potent effect of the cottonseed meal over that of the cottonseed flour.

The analysis for aflatoxin will detect levels as low as 7 ppb. Therefore, the negative aflatoxin analysis of the cottonseed flour (table 25) may be interpreted as meaning either *0* or some amount less than 7 ppb. Thus, it is not known if the aflatoxin differences between the two cottonseed ingredients is relative or absolute.

Analysis of the total and free gossypol content (table 25) as well as of the thiophosphates and chlorinated hydrocarbons (table 26) do not indicate marked differences between the meal and flour components of the diet.

Other salmonids and cottonseed meal

The U.S. Fish and Wildlife Service's report, Summary of Trout Hepatoma [*2*], September 1960, and the Supplement, indicated that rainbow (*Salmo gairdneri*), cutthroat (*Salmo clarki*), and brown trout (*Salmo trutta*) were found with hepatomas. Hueper and Payne [*3*], Snieszko [*4*], and Ghittino and Ceretto [*5*] reported that brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) did not develop hepatomas when fed the same diet which produced hepatomas in rainbow trout. Because some of these reports stemmed from uncontrolled observations and were in conflict, we felt further study desirable.

In addition to the previously described work, a segment was included to test the DA (Santa Monica) diet on brown trout (*Salmo trutta*), Mt. Whitney strain; eastern brook trout (*Salvelinus fontinalis*), Mt. Whitney strain; king salmon (*Oncorhynchus tshawytscha*), American River strain; and the silver salmon (*Oncorhynchus kisutch*), Alsea River strain.

These species were treated in the same manner as the rainbow trout except that the number of fish of each species was started with 5,000. Difficulty in starting the brown trout in feeding caused an unusually high loss (25 per-

TABLE 26.—*Analyses of total diets (DA, DB, DC) for thiophosphates and chlorinated hydrocarbons*
[Analyses done by Stoner Laboratories, Campbell, Calif.]

Diet	Thiophosphates			Chlorinated hydrocarbons			
	Ethion	Baytex	Parathion	Total	DDT	DDE	Unknown
DA	0.07 ppm	0.04 ppm		0.15 ppm	0.05 ppm	0.01 ppm	0.04 ppm
DB	0.11	0.05	0.07 ppm	0.11			0.06
DC	0.13	0.05	0.05	0.14			0.04

TABLE 27.—*Hepatoma in salmonids on DA diet (20% cottonseed meal) by month of age and average weight, Darrah Springs, Calif., 1964–65*

Kind of fish	Age (in months)	# Hepatoma/ # examined	Average weight (grams)
Rainbow	5*	7/25	11.82
Rainbow	6	12/25	21.81
Rainbow	7	19/25	44.5
Easternbrook	7	3/20	57.0
Rainbow	8	19/25	66.4
Rainbow	9	22/25	120.6
Easternbrook	10	7/25	170.53
Brown	10	0/25	96.53
Silver Salmon	10	0/26	23.31
King Salmon	10	0/25	69.6

* No hepatomas seen before fifth month.

cent) during the first few weeks on the diet. The silver salmon were affected by white spot and about 10 percent were lost before swimming up. After 3 months of separate rearing all species were mixed in one nursery tank except the brown trout which were held separately for an additional 6 weeks. At the end of 10 months on the DA ration, liver sections of the four above species revealed only the brook trout to be positive for hepatoma (table 27). Although several brown trout livers have exhibited suspicious areas, none have been confirmed as hepatoma at this writing.

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Discussion

DR. JACKSON: Well, very briefly, I have no new results to add to the report we have here. We have no slides to show, but I would be very happy to attempt, to answer any specific questions that you might have concerning the work that we have described here. There may be needed clarifications or questions regarding the limitations in the work. I would like only to outline what we think to be our significant findings at this point in time. We are not through with these experiments. These findings merely indicate what we observed up to the time we prepared the material for this conference.

Basically what our observations mean to us at this point is that we were able to repeat the work that we did previously, indicating that cottonseed meal was associated with the occurrence of hepatoma. Also, cottonseed flour showed the same effect, although apparently at a lower level in terms of its potency. And last, it is apparent that this association is not a species specific phenomenon in that brook trout fed cottonseed meal are now showing gross hepatomas.

I think the biochemical work that has been done speaks for itself. It will have to in the sense that I am not involved in the biochemical work. Mr. Sinnhuber from Oregon did the aflatoxin and the gossypol analyses. The DDT and the other insecticide work was done by Stoner Laboratories. However, Mr. Sinnhuber may like to interpret the results of the aflatoxin analysis.

DR. HALVER: Would you care to comment on the extension beyond the actual trout hepatoma research? Your program is continuing as outlined here, but you also have other interests in the general area of aflatoxicosis and carcinogenesis, don't you?

DR. JACKSON: I have no long-term abiding interest in the aflatoxin prob-

lem. I don't see that in the United States, anyway, there is a clear indication that it presents a serious human cancer problem. My interest is in human cancer, and I can't see, at least in primary cancer of the liver, that there is a great field to explore.

I think the persons who are concerned with international health work, where indeed there are many populations with a very high incidence of primary liver cancer and a unique geographic distribution of these, that there is a problem to investigate. I suspect that if I were concerned with health problems in Africa, for instance, or in Asia, this would be a very interesting problem to pursue in terms of human disease.

My long term interest in the subject at hand is the use of fish as a biologic system for testing carcinogens more than the specifics of aflatoxin. And I am concerned about a number of innate problems in using fish, such as our failure to establish some reasonable standard conditions for handling fish so that we have some ability to compare results from one investigator to another.

I have been in this particular business for 5 years and there has been no area of agreement as to water temperatures. There has been no area of agreement as to some standard basal diet. There has been no agreement as to the development of isogenic strains of fish. If we are going to continue in this work, these are problems we should be concerned with. However, maybe this is peripheral to the content of the conference.

DR. HALVER: No, I don't think so. I think it's the current status of not only where we are in trout hepatoma research, but where we need to go next.

DR. JACKSON: I think we are pushing the point to try and say, "Be very careful in quantitation of dosage in these animals," if we are not equally concerned with the fact that we may be dealing with a very diverse genetic population with different propensities to respond to this agent. To be overly concerned with one without being concerned with the other, I think is to miss the point.

DR. SCARPELLI: Can you tell us a little bit about what criteria were used with the histopathology here? When you say "hepatoma" do you simply mean basophilic nodules, which some of us have reservations about really calling hepatoma?

DR. JACKSON: These were all diagnosed microscopically. Indeed, a great number of these are gross tumors as well, but most of the hepatomas in the early stages, that is at 5, 6, 7 months, would be what Dr. Simon described as grade C. Most of the hepatomas now seen in the rainbow trout are gross tumors and would be described by that classification as "D".

It's interesting that Dr. Simon showed two metastatic lesions. In addition to how these look under the microscope—admittedly not a highly malignant appearing tumor; the natural history of this disease in rainbow trout has shown a number of metastic lesions. So the concern with the histopathology is one, but the concern with the natural history of the disease is another one, and I don't think that we can say that this is a hyperplastic nodule if we are finding metastases in the kidney.

DR. GOLDBLATT: A question deals with your aflatoxin content of the cottonseed flour, which is zero with 7 as the detectable limit. Are there any plans to try to get it more accurately than that?

DR. JACKSON: Well, we would certainly like to and we would hope that the subsequent discussions today might indicate to whom we can turn to do this. I don't want to speak for Mr. Sinn-

huber, but I understand that he is refining his technique. I know Dr. Helwig in our own department is trying to set up to do these analyses, too. We do have meal set aside. It will be available for further analysis. And I think this is obviously a very critical point to determine and one that I don't think is open to interpretation at this time. We just don't know whether aflatoxin is there or not.

MR. SINNHUBER: I might comment on that. In our last conference in Berkeley we obtained the clean-up procedure which has been used by Pons, and we have not tried this to further see if we can do this more accurately. We probably could, and there is some interfering material. I might say we were unable to get a value on cottonseed flour. The plate was completely negative. It's been very difficult to assay the total diet containing something like 25 percent fish meal. This really is tough to clean up. Perhaps Dr. Goldblatt and Mr. Pons would be able to help us on this. We are trying it at the present time with your clean-up procedure, and we haven't been too successful. I believe Dr. Wogan might comment that he was able to clean up some commercial rations.

DR. WOGAN: Yes. Before I do I would like to ask a question about diet DA. This contains the cottonseed meal which had 36 parts per billion, is that correct?

DR. JACKSON: Yes, that is correct.

DR. WOGAN: We just finished an assay for aflatoxin on a total ration and found, in fact, on the order of 220 parts per billion in the total diet. So it may be a bit misleading to base the whole judgment on what one component might contain.

DR. JACKSON: Yes. I think it's interesting, however, that the DB diet, which is only different from the DA in the sense that it has had the entire cottonseed portion removed, continues to be negative for hepatomas. Again, we are at maybe a midpoint in this experiment. The same phenomena applied in our first work, which ran for 24 months, in which the diet without the cottonseed meal remained negative through all this period.

DR. LEE: You show that one other feature in common was that there was a tenth as much gossypol in the flour as there was in the meal. Has anybody done any work with just gossypol alone added to a diet which does not contain any cottonseed products otherwise?

DR. SCARPELLI: We fed gossypol in a synthetic diet, but with no cottonseed products in it at all except gossypol. These experiments have been going on now for about a year and 3 or 4 months, the fish are markedly stunted in their growth and are deeply pigmented. The livers are also pigmented, the pigment is olive green and resembles the olive green pigment gossypol. Thus far, no neoplasms or basophilic nodules have developed.

DR. GOLDBLATT: You say that was at 15 months?

DR. SCARPELLI: Yes.

INTERIM HEPATOMA RESEARCH REPORT

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In April of 1960, a shipment of live rainbow trout from a commercial trout hatchery in southern Idaho was routinely examined at the California border by representatives of the California Department of Fish and Game. These fish, from an area in Idaho, were found to be affected with hepatoma. Since there were indications that feed might be involved [*1*, *2*] as a possible causative factor, the Ralston Purina Company became quite interested in the problem. In order to determine whether cases of hepatoma might exist in other similar populations, the rainbow trout on experimental rations at our research unit were examined. These examinations did not indicate anything which, at that time, could have been diagnosed as hepatoma [*3*]. Therefore, if this condition might be attributed to the ration, it certainly was not universally present, and any possible causative factor in feeds must be a contaminant or in some way associated with certain feed components.

It seemed logical to approach the problem by feeding known compounds which might produce hepatoma. There was a paucity of literature in this field as far as trout are concerned, but since various workers in this area had postulated that certain ingredients might be involved, an experiment was set up to test some of the hypotheses.

Cottonseed meal, estrogens, and antioxidants were among the suspect materials, as were some of the trace elements. Trout hepatoma experiment 1 was designed to feed these materials with our commercial trout ration, Trout Chow, which had not, to the best of our knowledge, been known to produce any evidence of this disease. The variables used are listed in table 28 followed by the proximate analyses and the list of ingredients of the basal ration.

TABLE 28

Variable	Amount in ration
Diethylstilbestrol	0.5 mg./lb.
Dienestrol dincetate	31.75 mg./lb.
Cottonseed meal	39.65%
Selenium	0.1 ppm
Zinc	10.0 ppm
Zinc	5.0 ppm
Arsanilic acid	.01%
BHA	.0125%
Santoquin	.0125%

The following is a proximate analysis and ingredient listing of basal ration for hepatoma experiments 1 and 2:

	Percent
Crude protein (min.)	40.0
Crude fat (min.)	2.5
Crude fiber (max.)	5.5
Ash (max.)	13.0
Added minerals (max.)	3.0
Moisture (max.)	12.0

Ingredients: Fish meal, soybean meal, ground wheat, brewer's dried yeast, condensed fish solubles, ground yellow corn, dried whole whey, wheat middlings, dried beet pulp, animal fat preserved with BHA (Butylated Hydroxyanisole), 1.5 percent low fluorine rock phosphate, 1 percent iodized salt, vitamin A supplement, ascorbic acid, D activated animal

sterol, menadione sodium bisulfite (source of vitamin K activity), vitamin B-12 supplement, biotin, choline chloride, pyridoxine hydrochloride, thiamin hydrochloride, niacin, calcium pantothenate, riboflavin supplement, and traces of manganese sulfate, manganous oxide, zinc oxide.

Diethylstilbestrol and Dienestrol Diacetate have hormone activity and are compounds commonly employed in beef and poultry rations. Selenium has been used as a preventive of white muscle disease in lambs. Arsanilic Acid has been used as a growth stimulant for swine and poultry. BHA (Butylated Hydroxyanisole) and Santoquin are antioxidants used in feeds. Zinc is used in various rations to meet the requirements for this element. High level cottonseed meal was included in this group because of interest from a previous experiment.

One hundred thousand Nelson stock rainbow trout sacfry from the State of Idaho were randomly distributed into ten troughs and started on their respective rations. They received no other feed while on experiment. At the end of 330 days, the fish were examined for the presence of hepatoma. Only those fish with large, well-developed liver nodules were recorded as positives. Examples of these nodules were preserved in 10 percent formaldehyde and sent into our Pathology Department where hepatoma was confirmed, histopathologically. The results of hepatoma experiment 1 are listed in table 29.

The result of the first hepatoma experiment indicated that this cottonseed meal or an unknown factor in this cottonseed meal might be toxic to rainbow trout.

The results of experiment 1 were reported to the Research Committee of the National Cottonseed Products Association. The members were unable to offer any clues as to the possible carcinogen. As a result it was decided to screen all fractions of this cottonseed in hepatoma experiment 2. Assuming that the carcinogen was common to all cottonseed products, the sampling followed the normal order of processing as shown schematically in table 30.

TABLE 29
Dates of Experiment 11/4/60-10/1/61

Variable	Amount in ration	% Hepatoma
Diethylstilbestrol	0.5 mg./lb.	7.14
Dienestrol diacetate	31.75 mg./lb.	6.10
Cottonseed meal	39.65%	61.11
Selenium	0.1 ppm	negative
Zinc	10.0 ppm	negative
Zinc	5.0 ppm	negative
Arsanilic acid	.01%	negative
BHT	.0125%	negative
Santoquin	.0125%	negative

One of the authors followed this sampling from one lot of seed in quantities sufficient for this experiment and possible laboratory isolation later. The entire lot with the exception of the hulls, and meal were stored at 32° F. to retard any biological or chemical deterioration in storage. The tarry residue shown in table 30 is not employed in the food or feed industry and therefore was not included in this experiment.

One of the problems which has been worked on extensively by the NCPA Research Committee is the physiological effects of gossypol in cottonseed meal. Through the assistance of Mr. Edward Gastrock of the Southern Regional Research Laboratory of the USDA in New Orleans, we secured a concentrated source of these pigments in sufficient quantity for our needs. This was the miscella from the hexane-acetone-water extraction of cottonseed [4]. The solvent was removed from the miscella under a vacuum as needed to produce a very dark crude cottonseed oil rich in cottonseed pigments (see table 31, ration 9).

TABLE 30

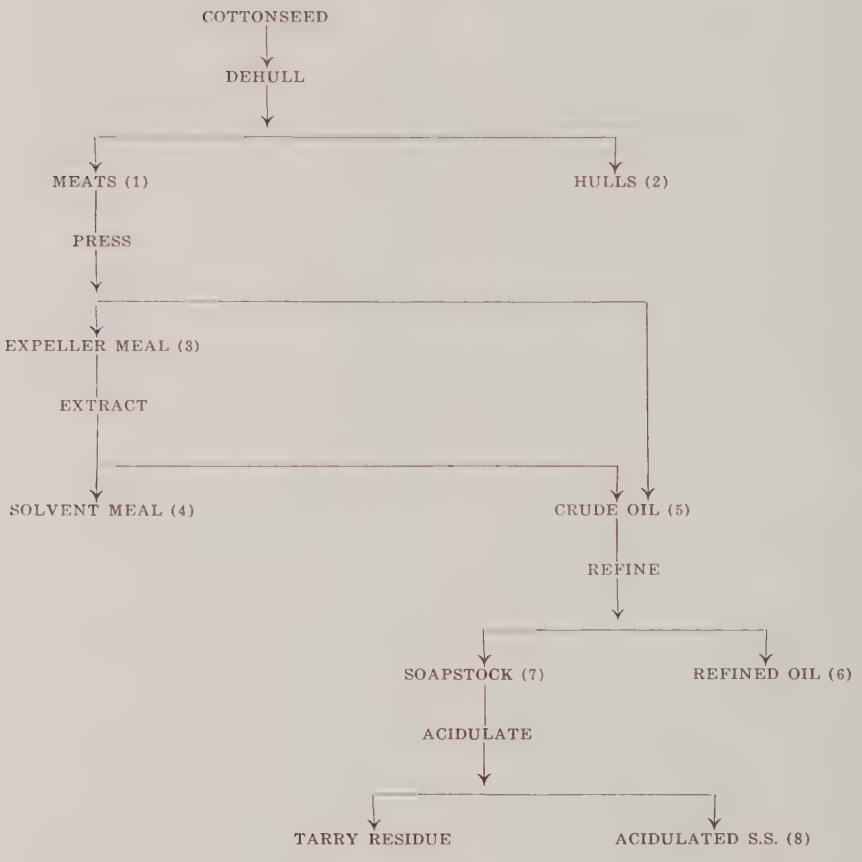

TABLE 31

Dates of Experiment 4/8/62–6/30/63

Variable	Amount in ration	% Hepatoma
1. Cottonseed meats	45%	negative
2. Cottonseed hulls	16	negative
3. CSM expeller	40	negative
4. CSM solvent	40	negative
5. Crude cottonseed oil	1	negative
6. Cottonseed oil refined	1	negative
7. Crude CS soapstock	4	negative
8. CS acidulated soapstock	2	negative
9. Crude CS oil extracted (H-A-W)*	2	negative

* Hexane-Acetone-Water extraction.

All cottonseed meal fractions were included in the ration in relation to their concentration in normal cottonseed meal and in relation to the amount of cottonseed meal (40 percent) in experiment 1. One hundred eighty thousand starting Nelson stock rainbow trout sacfry were randomly divided into troughs and started on their respective rations. The variables in the ration and the results of the hepatoma examination are listed in table 31.

These fish were kept on experiment for approximately 450 days with no evidence of liver tumors. These results suggested that either cottonseed products were not toxic or that the toxic factor was not present in all sources of cottonseed meal.

Unsuccessful attempts were made to trace down the cottonseed meal used in experiment 1 to the original source and manufacture. Cottonseed meal for

hepatoma experiment 2 came from the Mississippi Delta area.

This area was chosen because some seed is not stored under favorable conditions. In addition defoliants were employed on a large portion of the crop to permit mechanical harvesting of the cotton.

The possibility of aflatoxin as a causative agent was considered. The accepted assay, at that time, was the bioassay employing ducklings [5]. The bioassays on all the fractions were negative.

The first hepatoma experiment indicated that the cottonseed fed contained a carcinogenic factor. The second hepatoma experiment suggested that this factor was not present in all sources of cottonseed meal. The third hepatoma experiment was designed to survey various geographic sources, methods of processing and crop year of cottonseed. These meals were collected and coded by the National Cottonseed Products Association and shipped to the Ralston Purina Company's Production Research Department for subsequent manufacture of the experimental rations.

The basal ration for this experiment was the "Santa Monica" diet. This change in basal ration from the previous experiments was made to conform to the basal ration of other investigators [6].

The rainbow trout used in this experiment were the McCleary strain from the State of Washington and were from a different source than the previous experiment. From the start of the experiment 34,000 rainbow trout sacfry were randomly divided into 17 ponds and fed their respective rations. They received no other rations. At the end of 300 days on experiment the fish were examined and the results are listed in table 32.

The final examination of fish on hepatoma experiment 3 has not been made.

TABLE 32
Start of Experiment 3/16/64–

Cottonseed meals	Amount in ration	Aflatoxin B_1 in ppb in cottonseed meal (7)	% Hepatoma after 300 days
A	20%	Neg	Neg
B	20%	Neg	Neg
C	20%	250	57
D	20%	Neg	Neg
E	20%	4	Neg
F	20%	200	17
G	20%	190	30
H	20%	Neg	4
I	20%	Neg	Neg
J	20%	Neg	Neg
K	20%	25	Neg
L	20%	2	Neg
M	20%	Neg	Neg
N	20%	Neg	Neg
O	20%	8	Neg
P	20%	65	25
Soybean meal control	20%	Neg	Neg

During the course of the experiment, some of the outlet screens clogged thus raising the water level to a point where fish could have jumped from one pond to another. This might explain the positive tumors (4 percent) found in fish fed cottonseed meal H.

As cottonseed meal C, F, G, and P indicated positive results meals C, F, and G were discontinued, the fish being fed rations containing CSM P, soybean meal and the remainder of the cottonseed meals were transferred to the U.S. Fish and Wildlife Service hatchery at Hagerman, Idaho, for further study.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the National Cottonseed Products Association, especially Mr. Garlon Harper and Dr. Richard Phelps for their help in securing the cottonseed meal used in experiment 3; to Mr. Donald Rudnick for the management and care of the fish on test. We also want to express our appreciation to the Fish and Wildlife Service; especially Dr. J. Halver who allowed us to use facilities to carry on work to completion on experiment 3. We are also indebted to the attendees of the second annual hepatoma workshop conference for their suggestions in designing the third hepatoma experiment.

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NUTRITIONALLY INDUCED HEPATOMAGENESIS OF RAINBOW TROUT (*Salmo gairdneri*)

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Hepatoma in commercially reared rainbow trout (*Salmo gairdneri*) was first seen at this laboratory in April of 1960. The subsequent discovery of it in near epizootic proportions in other hatchery-reared rainbow trout and cutthroat trout (*S. clarki*) populations throughout the United States precipitated extensive research by numerous agencies. Although the liver neoplasm in trout had been previously noted here (Haddow and Blake, 1933; Nigrelli, 1954; Nigrelli and Jakowska, 1955) and abroad (Cudkowicz and Scolari, 1955) occurrences of it in such proportions had not been reported previously in this country.

History and histopathology of this hepatic tumor occurring since 1960 are well documented (Ghittino, 1961; Heuper and Payne, 1961; Nigrelli and Jakowska, 1961; Rucker, Yasutake, and Wolf, 1961; Snieszko, 1961; Wood and Larson, 1961; Ashley and Halver, 1963; Scarpelli et al., 1963; Wolf and Jackson, 1963).

The Branch of Fish Hatcheries, Bureau of Sport Fisheries and Wildlife, in March 1960 had started a feeding trial to evaluate four commercial dry pellets (diets A through D) at the Hagerman National Fish Hatchery, Hagerman, Idaho. Diet A was suspected to be a hepatoma-inducing diet. This paper describes the hepatomagenesis in the above hatchery-reared rainbow trout. Histological changes observed after removal of the hepatoma-inducing diet also are discussed.

Table 33 is the case history of the experimental fish. Tables 34 and 35 summarize the hepatomagenesis of the groups fed the various diets. Approximately 1,200 livers were processed and examined over a period of 18 months. Histopathological descriptions used in this report are in accordance with the National Cancer Institute standards of trout hepatoma nomenclature.

DIET-A GROUP

Bizarre changes of the liver parenchymal cells were observed in the initial samples and persisted throughout the experiment. The degree of diffusion, hyperchromatism and pleomorphism varied from several cells to multicell involvement. Eosinophilic, basophilic and anaplastic nodules were morphologically similar to those previously described by other investigators. At the 14th month this group was subdivided into three groups: group A was continued on the original diet; groups A_1

TABLE 33.—*Case history of experimental rainbow trout*

1/22/60	Eyed-eggs received from the Oregon Game Commission hatchery in Scio, Oregon.
2/13/60	Started feeding frozen beef liver.
3/2/60	Split into four groups and started feeding each a commercial pellet mixed with beef liver.
3/7/60	Started on 100% commercial pellets.
6/28/60	First sample received for histological examination.
4/61	A-group divided into three groups and started on A, A_1, and A_2 diets.
12/11/61	Last samples received for histological examination and experiment terminated.

TABLE 34.—*Summary of hepatomagenesis in rainbow trout fed diet A*

Month on diet	A				A_1				A_2			
	Bizarre	Eosinophilic nodule	Basophilic nodule	Anaplastic nodule	Bizarre	Eosinophilic nodule	Basophilic nodule	Anaplastic nodule	Bizarre	Eosinophilic nodule	Basophilic nodule	Anaplastic nodule
4	1/10*											
5			2/10									
6	3/10											
7			1/10									
8	7/10		3/10									
9	5/20	4/20	6/20									
10	7/20		5/20									
11	4/20	4/20	5/20									
12	1/20		6/20	2/20								
13		5/20	5/20	1/20								
14		6/20	6/20	2/20		2/20	5/20	2/20		7/20	11/20	2/20
15		3/20	7/20	3/20			10/20	2/20		1/20	6/20	7/20
16		1/10	3/10			5/10	6/10	1/10			5/10	2/10
17		1/10	6/10		1/10	2/10	3/10	1/10		1/10	1/10	5/10
18		4/10	5/10	2/10		3/10	5/10			2/10	8/10	1/10
19		2/10	3/10	2/10		5/10	5/10	2/10	1/8	4/8	4/8	
20	1/10	2/10	6/10	2/10			2/10	2/10		4/10	4/10	4/10
21		2/10	8/10	1/10	1/10		5/10	2/10		2/10	3/10	4/10

* Number diseased/number examined.

and A_2 were fed diets considered to be non-hepatoma inducing. There was no evidence of hepatoma-regression in any of these subgroups. Cholangiomatous changes occurred in six livers taken after the 11th month. Two spleen and one gill metastases were found in the 18-, 19-, and 20-month samples, respectively.

DIET-B AND DIET-D GROUPS

With the exception of often extensively vacuolated hepatic parenchymal cells in the B group, no significant pathology was observed in these two groups.

DIET-C GROUP

Cytopathology similar to the earlier specimens of the A group first became apparent in the 11-month samples. Advanced hepatomatous changes in this group were conspicuously lacking; however, bizarre changes and basophilic nodules were observed. Eosinophilic forms were seen only in one liver.

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TABLE 35.—*Summary of hepatomagenesis in rainbow trout fed diets B, C, and D*

Month on diet	B			C			D		
	Bizarre	Eosinophilic	Basophilic	Bizarre	Eosinophilic	Basophilic	Bizarre	Eosinophilic	Basophilic
4									
5									
6									
7									
8									
9									
10									
11				2/20*		1/20			
12				4/20		1/20			
13				3/20	1/20	1/20			
14						1/20			
15									
16									
17						2/10			
18				5/10		1/10			
19				2/10		1/10			
20				1/10		1/10			
21				7/10		2/10			

* Number diseased/number examined.

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Discussion

MR. YASUTAKE: Dr. Simon's discussion this morning has made my task of discussing our pathogenesis material a little easier. Before we go into the slides, I would like to give a little background on the material that we used for our experiment.

We first observed hepatoma in rainbow trout in April of 1960. These initial samples of livers were sent to us for diagnosis by a commercial fish farmer. Shipment of his rainbow trout had been refused entry at the California border by the California Department of Fish and Game because many small "pustules" were found on the livers of a number of fish.

Subsequent investigation by Dr. Rucker, Mr. Hal Wolf, and myself included visits to various hatcheries in California. We found widespread evidence of hepatoma which we strongly suspected to be nutritionally induced.

Fortuitously, we learned shortly thereafter that the Branch of Fish Hatcheries in our Bureau had initiated at their Hagerman Hatchery in Idaho, a feeding trial in order to evaluate the growth of fish on each of four commercial dry pellets. With their kind cooperation we were able to obtain these materials. Feeding trial of these groups A, B, C, and D had been started in March 1960. Group A was being fed the diet which was suspected to be hepatoma-inducing, therefore we used this as the positive control.

By April of 1961, after 13 months of feeding, fish in groups A and C were found to have hepatomatous lesions. Since the greatest incidence of hepatoma occurred in group A, it was divided into two additional groups so that we could follow the hepatomagenesis after the removal of the toxic diet. Group A was continued on the same diet: A_1 was placed on a beef liver diet and A_2 on another commercial pellet considered to be nonhepatomagenic.

We divided our histopathological findings into 5 types:

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Type I—Normal
Type II—Bizarre form which is similar if not identical to the preneoplastic dysplasia previously discussed by Dr. Simon.
Type III—Eosinophilic nodule
Type IV—Basophilic nodule
Type V—Anaplastic nodule

Figure 2 shows a normal liver with the usual sinusoids, triad area, and cords two cells thick.

Figure 3 is liver from a group B fish which had been on diet for 19 months. This vacuolated liver condition persisted in this group throughout this experiment. Note the extensively vacuolated parenchymal cell. We were able to demonstrate glycogen with PAS stain of these livers.

Figure 4 is liver from the A group showing type II form, the bizarre or preneoplastic dysplasia, with pleomorphic and hyperchromatic nuclei. We found in measuring these large bizarre nuclei that they could be as large as 20 microns. Normally their diameter may vary anywhere from 4.6 to 6.3 microns.

Figure 5 is the earlier eosinophilic nodule we found in group A fish that had been fed for 13 months. You notice the architecture of the nodule is very similar to a "normal" liver. The plates are usually two cells thick. On first observation you may get the impression that these cells and nuclei were larger or swollen when compared to the "normal" cells. We found that the nuclei of this and also the earlier basophilic nodules are very similar.

DR. ASHLEY: On these eosinophilic nodules, do you notice the parenchymal

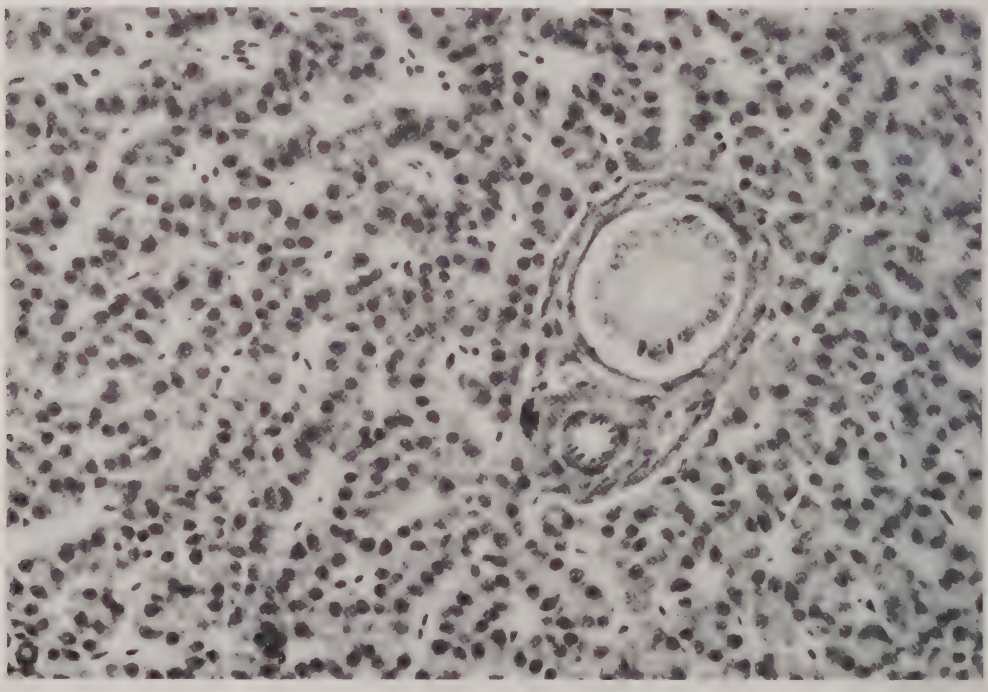

FIGURE 2.—Normal Liver. Note the prominent sinusoids and cords characteristically 2 cells thick. × 960.

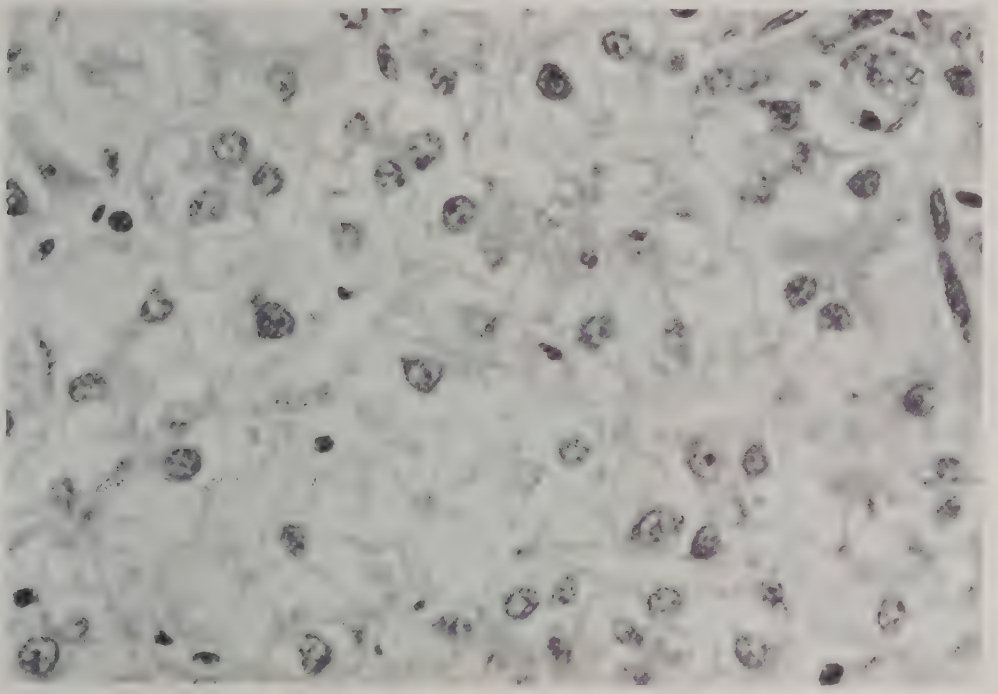

FIGURE 3.—Liver from fish in group B with extensive vacuolation of parenchymal cell cytoplasm. × 2160.

cells customarily are swollen, somewhat larger than in the rest of the normal liver?

MR. YASUTAKE: We did take measurements and found that nuclei frequently measured about the same, however the whole cell, that is when the cytoplasm is included, was larger than the "normal" cells. In larger eosinophilic nodules the nuclei were larger.

DR. ASHLEY: Do you ever find definitely widened liver cords or muralia in the eosinophilic nodules?

MR. YASUTAKE: In the larger nodules, yes.

Figure 6 is a magnification of the other nodule. The earlier basophilic nodules are very similar to it except for the basophilic staining factor. Note the cords two cells thick. When the "normal" cells are vacuolated like this, the nodules are very conspicuous because they usually lack the vacuoles.

Figure 7 shows atypical basophilic cells. We believe that this could be a regenerating form. Note the very small basophilic area here. This is atypical in the sense that these cells are very small and compact. These basophilic cells appear to be a little more compact than the "typical" early basophilic nodule cells. The intracellular vacuoles seen in some of these cells appear to be lipid vacuoles.

You will note that the morphology is quite different from the acidophilic or the "normal" tissues. These muralia are many cells thick and the nuclear sizes are very similar to those of "normal," but the cell proper is very compressed. We found mitotic

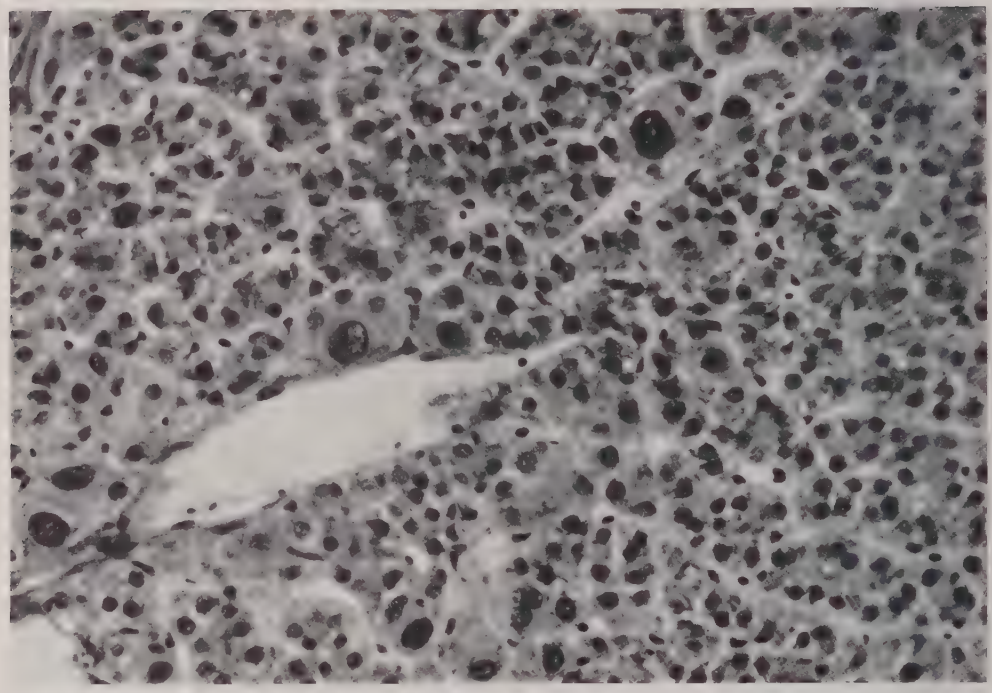

FIGURE 4.—Bizarre liver from fish in group A which had been on the test diet for 10 months. Note the large pleomorphic and hyperchromatic nuclei. × 1080.

figures only infrequently in most basophilic nodules. Note the thickened plate and the compressed parenchymal cells (fig. 7).

Figure 8 shows liver taken from group A which had been on the diet for 19 months. This shows the advanced form of basophilic nodule with a cholangiomatous area within the neoplastic tissue. Fibrous stroma may be seen between the ductules (fig. 8).

Figure 9 is splenic metastasis found in the 19-month fed fish in group A. In this particular tissue we observed two small, well differentiated and discrete nodules obviously of hepatic origin.

All the diets A, B, C, and D were commercial diets.

DR. SIMON: You think eosinophilic nodules, then are neoplasms . . .

MR. YASUTAKE: Are neoplastic, yes. We consider them to be, but we really don't know. As a matter of fact, we found a lot of eosinophilic nodules in the diet A Group, but in the C group we found only one.

DR. HALVER: Aren't pathologists challenged with the decision of what is it at the moment of examination, whereas the biologists and physiologists are concerned with what will happen to this in a parallel group, or if it were not sampled, what would have happened to it? It must remain speculative. We can only draw an analogy between what happens in a parallel group and what you see at particular times. I think that when Dr. Ashley outlines some of our early, intermediate, and advanced studies we may get more information on this point.

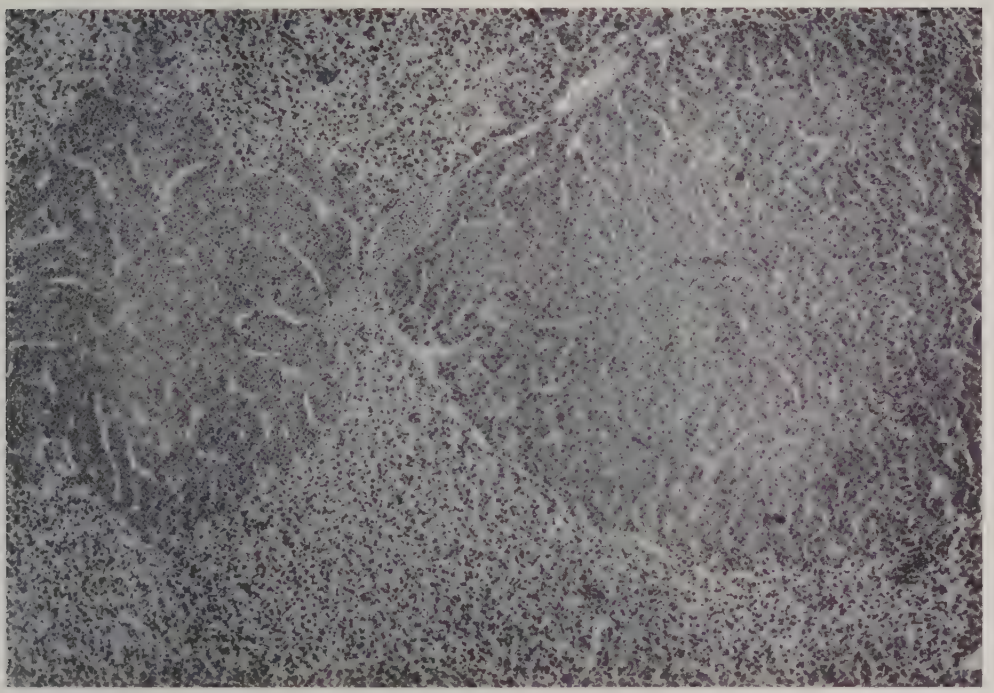

FIGURE 5.—Eosinophilic (top) and basophilic (bottom) nodules occurring in liver from fish of group A. It was fed the test diet for 14 months. × 192.

DR. SCARPELLI: One interesting point with reference to the eosinophilic or acidophilic nodules is that in the very early papers of Dr. Opie who did classical work on hepatomas induced with butter yellow, showed and described considerable numbers of eosinophilic nodules and concluded that these preceded basophilic nodules.

In the paper we published on this we have a photograph showing a single nodule, half of which is eosinophilic, and the other half is basophilic.

Bruni and Porter at the Rockefeller Institute, have shown that butter yellow in the case of the rat produces a very curious proliferation of smooth endoplasmic reticulum in the liver cells, which appear markedly eosinophilic when stained with the hematoxylin and eosin stain. This precedes the proliferation of granular endoplasmic reticulum ER, which parallels or accompanies the neoplastic transformation. I think these changes are extremely important in our understanding of hepatic carcinogenesis.

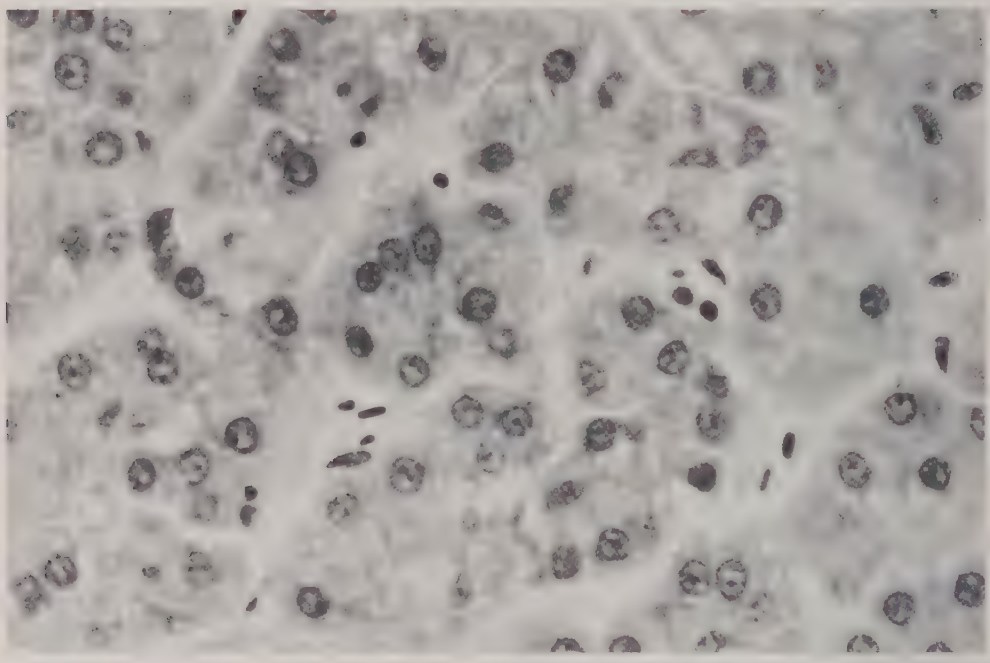

FIGURE 6.—Eosinophilic nodule in figure 5. Note that it has a comparatively normal morphology. × 2160.

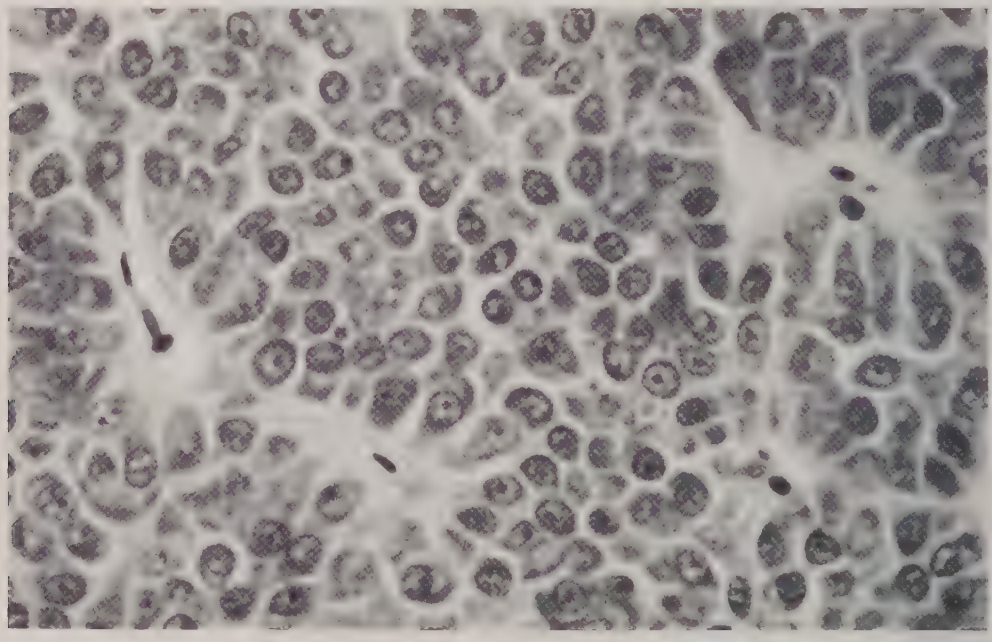

FIGURE 7.—Basophilic nodule in figure 5 showing the multicellular muralia and the compact parenchymal cells. × 2160.

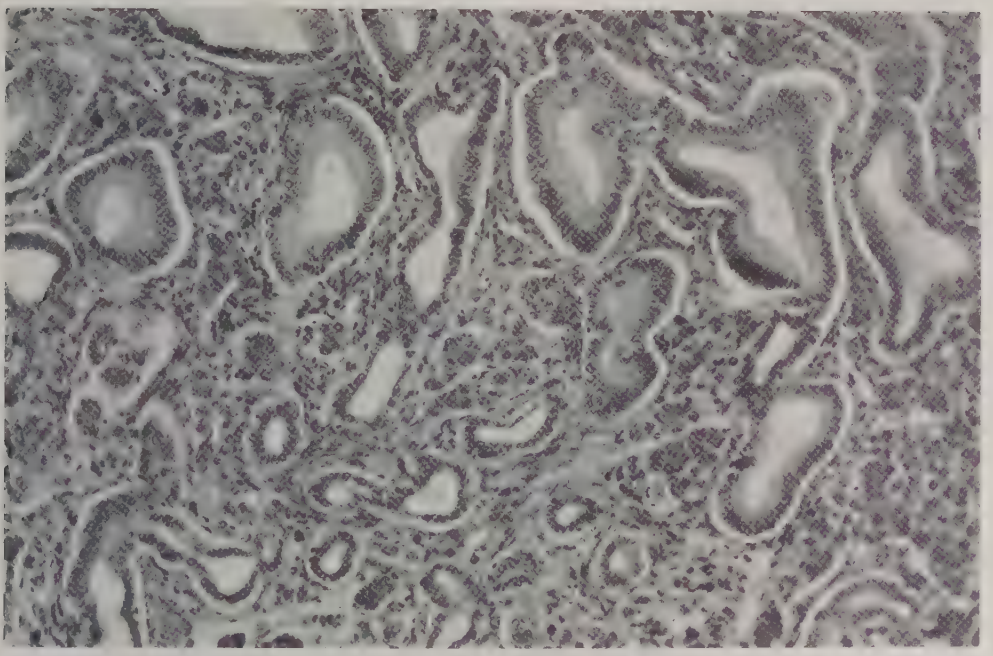

FIGURE 8.—Cholangiomatous area within a large basophilic nodule of a liver from group A fed the test diet for 19 months. × 480.

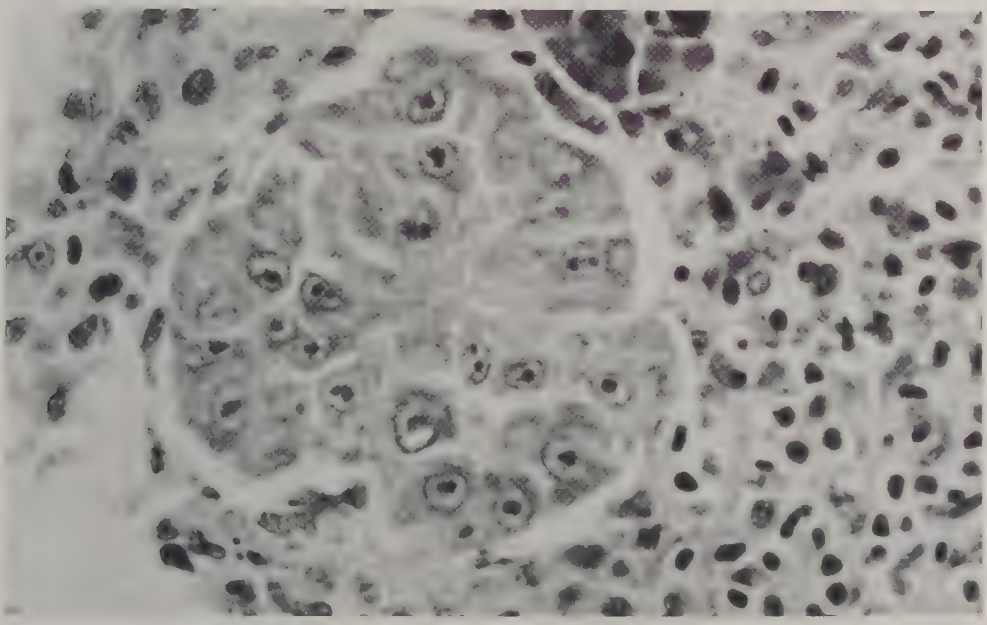

FIGURE 9.—Metastasis found in the spleen from fish in group A. It received the test diet for 19 months. × 2160.

AFLATOXIN IN COTTONSEED MEAL AND LIVER CANCER IN RAINBOW TROUT

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In 1960 an epizootic of liver cancer occurred in hatchery reared rainbow trout in the United States and Europe. Consideration of possible factors indicated that it was probably of dietary origin and the outbreak seemed to be correlated to the shift, in very recent years, from a diet of raw meal and fish materials to one of dry pelleted fish and vegetable meals.

A report by Wales [*1*] of observations made in 1935 has recently been prepared for publication and describes in detail the occurrence of liver lesions in rainbow trout which was attributed to a ration change from one of wet ingredients to dry fish, cottonseed and wheat meal.

Liver sections prepared in 1935 compare in detail with the rainbow trout hepatoma found in 1960. It can be stated with certainty that solvent residues and pesticides, two factors given careful scrutiny after the 1960 incidence were not involved in 1935. Solvent extractions of meals did not begin until about 1945, and DDT and similar pesticides were laboratory curiosities prior to 1943.

Fish meal and cottonseed meal seemed to be the two common denominators in all commercial rations that were associated with trout hepatoma. In addition the dry rations all contained lipids which have undergone severe oxidation.

Wolf and Jackson, in 1963 [*2*], demonstrated that a commercial ration, the SM ration, would produce hepatoma in rainbow trout but this did not occur when cottonseed meal was eliminated from the ration. They also fed cottonseed meal with beef liver and produced a significant incidence of liver lesions. It was concluded that cottonseed meal was responsible, but whether the carcinogen was intrinsic or extrinsic to cottonseed meal was unknown. Gossypol and cyclopropenoid fatty acids, both naturally occurring in cottonseed, have been shown to cause undesirable toxic physiological reactions in poultry and certain domestic animals when fed in sufficient quantities. Rainbow trout being a highly inbred strain, might react unfavorably to these components.

Halver and his associates also produced hepatoma by feeding a commercial ration, not the SM ration. Whether or not the ration contained cottonseed meal was not stated. They demonstrated that solvent extraction of the commercial ration concentrated the cancer producing material in the lipid extract. It was hypothesized that altered and oxidized lipids which are plentiful in dry trout rations could have a role in trout hepatoma.

During this period other investigators were involved in another problem concerned with an outbreak of liver cancer in turkeys which was traced to peanut meal and presence of a mold, *Aspergillus flavus* (Link). However, peanut meal was not an ingredient of trout rations, at least in America. It is

of passing interest that in the first trout hepatoma work session in April 1962, no mention was made of molds being a possible cause of hepatoma in trout.

Kraybill and Shimkin [*3*] in their recent article on carcinogenesis related to foods have reviewed the trout hepatoma problem and the role of fungal metabolites in diet and cancer.

In Oregon, we purposed to investigate the problem of hepatoma in trout and emphasize the role of lipids in the SM trout ration as possible factors in trout hepatoma. Our experiments were designed to first screen or test a number of common denominators that were present in the commercial ration (SM) with special attention being given to fish and cottonseed meal and lipid fractions. The SM ration was formulated and each ingredient of the ration was held for subsequent experiments if required. The composition of the commercial ration (SM) is given in table 36. The cottonseed meal present in this formula was a pre-pressed solvent extracted meal and is designated as the J cottonseed meal (J CSM).

The Oregon Test Diet (OTD), [*4*], which is a purified ration suitable for rearing salmon and trout, has been used by us for the past 12 years in evaluating potential food ingredients which might be used in rations for rearing salmon and trout in the production hatcheries of the Northwest. The basic formula of the OTD ration is also shown in table 36. An ingredient to be tested is first chemically analysed and then substituted at the desired test level in the OTD. Protein, lipid, minerals, or carbohydrate are removed from the OTD formula resulting in a test ration that is isocaloric and isonitrogenous to the original OTD ration. In a like manner, several of the components of SM ration were tested and in addition lipid extracts, special meals and compounds

TABLE 36

Commercial ration—SM

	Percent		Percent
Wheat middlings	25	Distillers' solubles	4
Fish meal	21	Dried skim milk	3
Cottonseed meal	20	Blood meal	3
Meat meal	6.6	Salt	2
Liver meal	6.6	Alfalfa meal	0.7
Brewer's yeast	6.6	Vitamin premix	1.7

Oregon test diet—OTD

	Percent		Percent
Casein	49.4	Salts	6.9
Dextrin	15.6	Carboxymethylcellulose	1.3
Corn oil	13.9	$CaCO_3$	0.9
Gelatin	8.7	Vitamin mix	3.6

such as gossypol, cyclopropenoid fatty acids and aflatoxins were incorporated in the OTD.

Rainbow trout eggs, artificially spawned on a single day, were obtained in December 1963, and the fish after hatching in January were given their initial feeding with the particular ration being tested. Three hundred fish each were held in 200-gallon fiberglass, circular tanks supplied with continuous flow of well water at a constant temperature of 53° F. with an oxygen content of 8.5 to 9.5 ppm. All lots were in duplicate. The diets were fed at 90 percent of the *ad libitum* amount. Each lot received only the diet indicated for the entire experimental period. The feeding frequency varied from 6 times each day when the fish were small; to once a day, when they became several inches in length.

After 6 months on the diet, a sample, representing approximately 10 percent of the fish in each tank, was taken, using a specially designed net for random sampling. The fish were anesthetized with tricaine methane sulfonate, explored for gross abnormalities, and placed in Bouin's fixative. A representative sample of the livers in each lot was embedded in paraffin, skip-sectioned, and stained with hemotoxylin

TABLE 37.—*The occurrence of hepatoma in rainbow trout fed a commercial ration and certain modifications*

Diet No.	Description	Hepatoma incidence						Aflatoxin level ppb*
		6 Months		9 Months		12 Months		
		A	B	A	B	A	B	
C1	Santa Monica ration (SM)	5/10	5/10	20/20	24/24	20/20	20/20	37
C2	SM without fish meal	1/5	-----	-----	-----	14/20	19/20	37
C3	SM without J CSM	0/9	0/10	1/9	3/10	2/8	2/10	(+)
C4	Diet 3 w. N CSM	2/11	-----	-----	-----	16/20	18/20	14
C5	SM (solvent extracted)	6/10	-----	-----	-----	17/21	19/20	(+)

CSM—cottonseed meal.

* As aflatoxin B_1.

and eosin. Samples were also taken after 9 and 12 months on diet, samples will be taken at 15 and 20 months.

The commercial ration (SM) and certain modifications are listed in table 37. The N cottonseed meal substituted for the J cottonseed meal was an expeller meal which had not been solvent extracted.

The lipid extracts, used in these experiments were made by exhaustively extracting a particular meal or ration with a 1:3 ethanol-diethyl ether, the solvents were removed with a rotary evaporator, under vacuum. The lipid extracts were substituted for the corn oil in the OTD at the level indicated.

The OTD and various combinations are listed in table 38.

Ashley, Halver, and Wogan [*5*] in 1964 reported that aflatoxin fed in a purified ration would induce liver cancer in rainbow trout at levels in the parts per billion range. Solomon [*6*] in work soon to be reported fed "toxic" peanut meal in a commercial ration to rainbow trout found a significant occurrence of liver carcinoma.

During the time our diet experiments were in progress we examined various procedures that were being used to determine aflatoxin in peanut meals and found them to be unsuited for cottonseed meals. A method was then devised for the determination of aflatoxin in cottonseed meals and this work has been submitted for publication. The method is a solvent extraction procedure using hexane to remove lipid, followed by acetone to extract the aflatoxins. Fractionation in the cold with methanol and chloroform is used to re-

TABLE 38.—*Occurrence of hepatoma in rainbow trout fed a purified ration and certain modifications*

Diet No.	Description	Hepatoma incidence						Aflatoxin B_1 level ppb
		6 Months		9 Months		12 Months		
		A	B	A	B	A	B	
1	Oregon test diet (OTD)	0/10	0/9	0/10	0/5	0/5	-----	0
2	OTD w. fish meal	0/12	-----	-----	-----	0/9	0/7	0
3	OTD w. J CSM	1/10	0/7	4/8	4/6	11/20	9/20	36
4	OTD w. N CSM	2/10	-----	3/8	1/7	13/20	8/20	15
5	OTD w. CS flour	0/7	0/7	0/6	0/5	0/5	0/5	0
6	ODT w. glandless CSM	-----	0/10	-----	-----	0/20	0/20	0
7	ODT w. fish meal lipid	0/10	-----	0/5	----	0/11	0/8	0
8	OTD w. J CSM lipid	-----	1/10	2/5	4/7	13/32	15/20	15.3
9	OTD w. N CSM lipid	0/9	0/5	0/5	0/5	8/20	7/20	3.7
10	OTD w. Santa Monica lipid	4/8	2/10	-----	-----	17/20	20/20	(42.8)
11	OTD w. Sterculia oil	0/10	-----	0/7	----	0/8	0/5	0
12	OTD w. gossypol (2000)	0/5	0/5	-----	-----	-----	-----	0
13	OTD w. gossypol (1000)	0/11	-----	-----	0/8	-----	-----	0
14	OTD w. peanut meal	1/11	1/12	2/5	3/7	-----	-----	8
15	OTD w. aflatoxin	0/5	1/5	0/5	1/7	3/6	2/6	8
16	OTD w. aflatoxin	-----	0/8	-----	1/8	-----	-----	4
17	OTD w. aflatoxin	-----	0/7	0/8	----	-----	-----	0.8

move interfering substances followed by thin layer chromatography. We are greatly indebted to Dr. G. Wogan and his associates at MIT for his kindness in providing us with aflatoxins for our experiments and for use as a standard in our assay procedure.

In tables 37 and 38 are presented, in ppb, the aflatoxin content of the various diets, in some cases the amount of aflatoxin was a calculated value based on the level of aflatoxin in the particular meal in that ration.

The level of gossypol in ppm is also given in table 38. The amount of gossypol in the various cottonseed meals was determined by chemical means. The cyclopropenoid fatty acid function in trout rations was determined by incorporating *Sterculia foetida* in the OTD.

Glandless cottonseed meal was generously provided by Mr. Garlon Harper of the National Cottonseed Producers Association. This meal is free of gossypol but does contain cyclopropenoid fatty acids.

The cottonseed flour was food grade quality and is used by the food and baking industry.

The peanut meal was supplied by Dr. G. Solomon and was part of a sample of toxic meal from Brazil.

RESULTS AND DISCUSSION

The incidence of hepatomas in rainbow trout fed the SM ration and certain modifications are present in table 37. The C_1 ration produced a high incidence of liver cancer after 6 months which was detected by microscopy. At 9 months the livers showed a 70 percent incidence of gross macro nodes; and at 12 months a sample had a 100 percent incidence of gross hepatomas. These results confirm the previous findings of Wolf and Jackson. It is of interest that the SM ration used in these experiments, although it was formulated from products purchased in the market more than a year after the diet was formulated for Wolf and Jackson, still contained a substance capable of inducing liver cancer in rainbow trout.

When the J cottonseed meal was replaced by the N cottonseed meal (diet C–4) it did not greatly affect the incidence of hepatoma despite the lower aflatoxin level. In C–2 when fish meal was replaced with casein, corn oil, and minerals, a somewhat lesser incidence of hepatoma was noted. Solvent extraction of the SM diet (C–5) was not effective in removing the carcinogen, but the incidence was reduced. Since acetone has been shown to be an excellent solvent for aflatoxin, it will be used in future extractions.

The incidence of hepatoma that still occurred when cottonseed meal was removed from the ration (C–3) indicates that the SM ration still contains active material, other than cottonseed meal, capable of inducing liver cancer in rainbow trout. We are unable to state at this time (2–24–65) which dietary ingredient is responsible or the nature of the substance involved. Work is in progress which we hope will provide the answers to this new problem.

The occurrence of hepatoma in rainbow trout fed the OTD and various modifications for 12 months are shown in table 38. No hepatomas were observed in trout on the OTD (diet 1) and the livers have a normal histology. This was also the situation with fish fed the OTD with fish meal (diet 2) or the fish meal lipid (diet 7).

Trout fed the OTD with J (diet 3) or N (diet 4) cottonseed meal showed microscopic liver lesions at 6 months and by 12 months had a 50 percent incidence of gross trabecular hepatomas. The lipid extracts of the J (diet 8) and N (diet 9) cottonseed also pro-

duced a significant incidence of hepatomas. A feeding test, employing ducklings, confirmed the results of the trout trials and the chemical tests. It is of interest that levels of aflatoxin as low as 3.7 ppb (diet 9) produced a 38 percent incidence of gross hepatomas in 12 months.

The lipid extract of the SM ration (C–1) replaced all the corn oil in the OTD (diet 10). The calculated level of aflatoxin, based on that present in the J meal, would be 42.8 ppb if fed at 2.8 times the lipid content of the SM (C–1) ration.

Cottonseed flour used as a supplement in the OTD (diet 5) produced no hepatomas and the livers showed a normal histology. Cyclopropenoid fatty acids and gossypol were present. A similar result was obtained on trout fed the glandless cottonseed meal (diet 6).

The OTD ration (diet 11) containing sterculic acid also did not induce hepatoma in trout after 12 months.

The OTD ration to which were added 2000 ppm gossypol (diet 12) produced a high mortality. A level of 1000 ppm (diet 13) was tolerated and no hepatomas were found at this level.

The diet which contained the toxic peanut meal (diet 14) produced microscopic liver lesions in 6 months and a 42 percent incidence of gross tumors by 9 months.

The OTD with varying amounts of aflatoxin are designated diets 15, 16, and 17; the incidence of hepatoma is shown in table 3. Based on these very preliminary results, it appears that approximately 1 ppb aflatoxin may be near the no-response level in rainbow trout fed the purified ration described in this report.

It may be concluded from the results of these experiments that cottonseed meal containing aflatoxin is responsible for the hepatoma in rainbow trout fed the SM commercial ration. This was demonstrated by chemical analysis, by duckling tests and by feeding trials with rainbow trout. At least one more carcinogen appears to be present in the SM diet which is unknown at the present time. Other factors seem to be involved such as altered or oxidized lipids. These may act as co-carcinogens or promoting agents.

Cottonseed flour and glandless cottonseed meal as well as cyclopropenoid fatty acid and gossypol did not produce hepatoma when fed in a purified ration (OTD).

ACKNOWLEDGMENT

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Discussion

MR. SINNHUBER: I think first, before time gets away from me, I would like to acknowledge the many people that have contributed to this work. There have been many people, including Professor Wales, Dr. R. Engebrecht, Don Amend, and Walt Ashton of our staff, Dr. G. Wogan contributed the

aflatoxin for this work, and Dr. Goldblatt provided us with gossypol, and many other people. This work is sponsored by the National Cancer Institute.

Because of the shortage of time, I am not going to go through our entire experimental design and the results we obtained. It is presented in the paper. I would just like to comment on one or two things, if we could go to the tables.

I think really what we started out to do was to reproduce the ration used by Wolf and Jackson and to attempt to confirm their findings, more than a year later. We also were interested in determining the contribution, if any, cottonseed meal had to the incidence of hepatoma in trout. As you know, there are some unusual compounds in cottonseed, such as cyclopropenoid fatty acids and gossypol, which were mentioned just a few moments ago. We are interested in studying these as well. At the time that we started our work I don't believe that aflatoxin was even being considered as a factor in trout hepatoma.

In reference to the tables, these data should be regarded as preliminary findings.

Regarding our experimental design, we are feeding commercial rations, including the Santa Monica diet, as it was described by Wolf and Jackson. Then we are substituting various ingredients and components of commercial rations in a purified ration. These are listed in table 36.

This purified diet is very similar to one that Dr. Halver uses. We have been using a test diet like this for many years to test or evaluate various diet ingredients.

Cottonseed meal, for example, is incorporated at a 20 percent level, the same level as in the Santa Monica diet, and then we add less casein, dextrin, etc., so that the final diet is isocaloric and isonitrogenous and is as close as we can make it to the original control diet.

We do subsample each group at predetermined intervals. We have worked out a method of sampling the lots and we think it is quite satisfactory.

Referring to table 2, the commercial ration listed as C–1, is the Santa Monica ration formulated a year after the work of Wolf and Jackson.

The two ingredients we were concerned with, and which seemed to be common to most commercial trout rations were cottonseed and fish meal. These were looked at first. We withdrew fish meal and still had a high incidence at 12 months.

The aflatoxin levels listed should be corrected on the basis of some very recent data we obtained only yesterday. We should raise the aflatoxin level to 43 parts per billion in ration C–1.

When we withdrew the cottonseed meal, the J cottonseed, which is the one in commercial ration, we still obtained an incidence. I will comment on this ration later.

But when we substituted another cottonseed meal, diet C–4, which I believe is the F expeller meal, it also produced hepatoma or an incidence of tumors.

We were also interested in the lipid fractions. Dr. Halver has been working in this area, and we proceeded to try to remove the carcinogen with ethyl ether and alcohol. We were unsuccessful in doing this. I think our work in the future will probably use acetone extraction, probably hot acetone.

Now returning to diet C–3, this ration does not contain cottonseed meal but does have all the other ingredients. We did not anticipate that we would have an incidence in this lot, however, we definitely are at the present time. We just completed our examination of more tissues and the incidence is over 50 percent.

We have run aflatoxin by chemical assays on all the diet components, and the one that appeared positive was wheat middlings. This has now been tested by duckling and the results were obtained only two days ago. It has been found to contribute to the ration about 6 parts per billion.

Therefore the level in the wheat middlings, assuming them to make up 25 percent of the ration would be 24 parts per billion.

I think it is interesting to go back and look at the work of Wolf and Jackson of more than a year ago, where they obtained a small incidence on wheat middlings. I won't comment on this except to say that we discussed this finding at some length at our meeting in Colorado, wondering as to the cause. Apparently now we have another feed material, in addition to cottonseed and peanut meal, which has been demonstrated to contain aflatoxin and will produce hepatoma in rainbow trout.

Referring to the diets on table 38, these are all substituted in the purified ration, and I will only comment on a few of them. As you can see, with the J cottonseed meal (diet 3) substituted in the purified ration we are obtaining about a 50 percent incidence of tumors. With the N cottonseed (diet 4) substituted in the purified ration the incidence is about 52 percent.

The cottonseed flour (diet 5) which was commented on earlier by Dr. Jackson has been found to be negative. The livers, as far as we can tell, look quite normal. The glandless cottonseed meal (diet 6) also was negative.

We also prepared some lipid extracts and added them to the purified test ration.

This was also done for diets 8 and 9. Fish meal lipid was negative and as you can see, the J cottonseed meal lipid and N cottonseed meal lipid were positive. Apparently our extraction, while still incomplete, did remove some aflatoxin.

Diet 10 is a test of the lipid extract obtained from the commercial ration. It also produced a very high incidence.

We were interested in the cyclopropene fatty acids because of their known physiological activity reaction in poultry and rats. This material was fed in the form of *Sterculia foetida* oil. Up to this time, we have not noted any incidence of hepatoma. The livers show some changes and we hope to do some more work on this point.

We fed gossypol at 2,000 parts per million in the purified action and we lost a considerable number of fish. We repeated the experiment at 1,000 ppm The fish are yellowish in appearance and the livers do show deposition of some unusual material. We are conducting histochemical tests on these animals. We have not encountered hepatoma in this group.

A sample of Brazilian peanut meal was provided by Dr. Gordon Solomon. He found it to produce hepatoma in trout when added to a commercial ration. We fed it in our purified ration and confirmed Dr. Solomon's findings.

I believe we have shown that gossypol fed in a purified ration did not produce hepatoma in rainbow trout, at least after 12 months. We are looking further into the cyclopropene fatty acids, and we are testing combinations of some of these materials with aflatoxin to see if they might act as cocarcinogens.

DR. NEWBERNE: What is the difference in the cottonseed meal in the flour and were these made from the same seeds?

MR. SINNHUBER: The meals are commercial feed grade. The J cottonseed meal was a pre-pressed solvent meal, obtainable as stock or animal feed. The cottonseed flour is prepared

and used for human consumption. It meets standards as set by the Food and Drug Administration.

DR. GOLDBLATT: If you have time, for No. 11 what was the proportion of Sterculia oil that you used?

MR. SINNHUBER: The cyclopropene function that was calculated was 200 parts per million as cyclopropene.

DR. KOTIN: In your table 38 you report data used in hepatoma incidence as the end point and in your text you say for those that showed no hepatomas, they were negative. Obviously they were negative in terms of hepatoma, but what about other signs of hepatotoxicity? Are your positive and negative exclusively in terms of hepatoma incidence or in terms of the liver showing no pathology at all, essentially non-neoplastic pathology?

MR. WALES: The figures that are given for hepatoma incidence mean just that, but in addition there is a great deal of liver injury, which usually accompanies hepatoma incidence, but not in all cases, as I will bring out later.

DEGENERATION AND REGENERATION OF LIVER PARENCHYMA ACCOMPANYING HEPATOMAGENESIS

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The commercial trout ration Santa Monica (SM) which has been tested in our laboratory during the past year has produced a clearly defined sequence of degenerative and regenerative changes in the rainbow trout liver. I propose to describe these and suggest their possible relationship to hepatoma.

Microscopic examination of livers was made after the fish had been on the commercial ration for 84 days. This was 94 days prior to the time the first hepatoma node was discovered. The parenchymal cells had undergone marked degenerative changes and regeneration of the secondary population was well advanced (fig. 10). This toxic degeneration of the primary generation of parenchymal cells and the concomitant development of the secondary generation has been followed through the 6-, 9-, and 12-month samples. These changes occur in diets which contain all of the commercial ration components, also in the Oregon Test Diet (OTD) to which certain portions of the commercial diet have been added, and in the OTD with added peanut meal. All of these diets contain aflatoxin in varying amounts and possibly other toxic substances.

The typical parenchymal cell undergoing degeneration apparently fails to divide in the normal manner, attaining a volume several times that of normal liver cells. Similarly the nucleus becomes greatly enlarged and eventually more or less irregular in shape (figs. 11, 12, and 13). At some point in the process of enlargement we assume that some intracellular metabolic system becomes impaired and the cell dies. The cellular membranes disintegrate, the cytoplasm in the sectioned tissue ap-

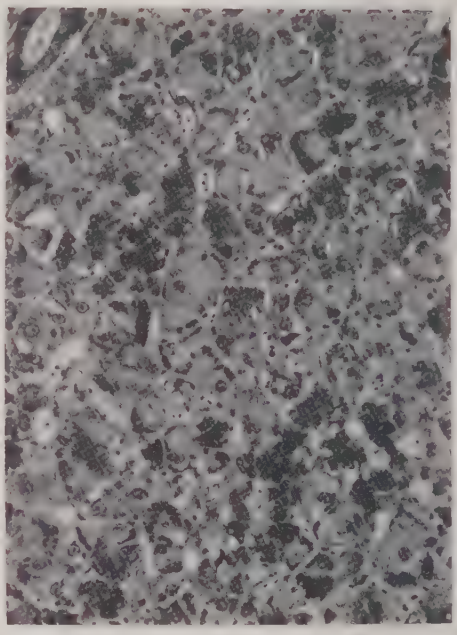

FIGURE 10.—Islets of regenerating trout liver parenchyma developing among degenerating cells of the original population. The regenerating cells are small and basophilic whereas the degenerating cells are abnormally large eosinophilic. H. & E. × 128.

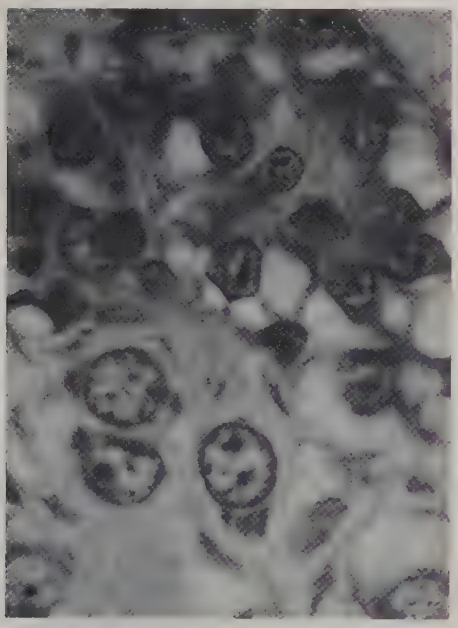

FIGURE 11.—An islet of regenerating parenchyma (lower right), and enlarged, degenerating cells (upper left). The sizes of the degenerating cells and their nuclei are much greater than in the regenerating tissue. H. & E. × 800.

pears granular and eventually disappears. Finally the nucleus may be left and finally it also disintegrates. The dying cells are scattered among those which are still able to carry on more or less normal processes. The death of these cells would appear to be a prolonged affair. While this is occurring one finds islets of small, basophilic cells starting to form. These increase in size and number and eventually replace the injured parenchyma. These conclusions are based upon observations of many livers of several diet groups taken at 4, 6, 9, and 12 months of age. The first step in the regeneration process is the appearance of a few clusters of small, basophilic cells contrasting sharply with the enlarged, eosinophilic parenchymal cells of the original population (fig. 11). In other livers we can find larger masses of these basophilic cells, and eventually the only evidence of the primary generation is an occasional giant, bizarre nucleus.

The tissue from which this secondary population arises is as yet unknown. They may have the same origin as the primary population. We will attempt to discover whether the secondary generation becomes injured in time by the toxicity of the diet or whether it remains immune as it grows older.

Observations of many hundreds of slides indicate that the islets of regenerated or secondary parenchyma do not appear to arise from ductal tissue even though the close association of the two might suggest this. Proliferation of trout bile duct and vascular tissue into surrounding parenchyma is beautifully

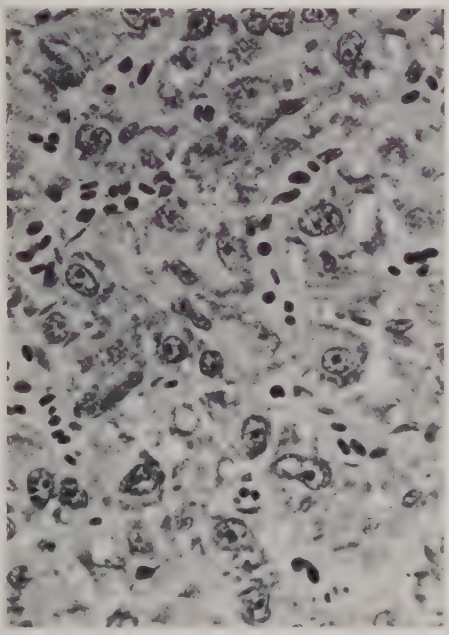

FIGURE 12.—The sizes and shapes of degenerating parenchymal nuclei are highly variable, in contrast to the normal trout liver nuclei, which are uniformly small and oval. H. & E. × 320.

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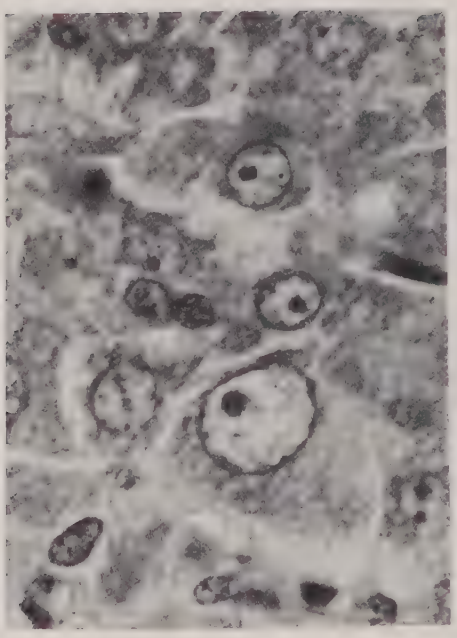

FIGURE 13.—The chromatin is more dispersed in the enlarged nucleus of the degenerating liver cell. H. & E. × 800.

displayed in the duckling, and although it does occur extensively in the trout it is not as easily evaluated. Also the rate of tissue growth in the duckling is normally in the order of four to five times as rapid as in the trout. For these reasons it does not appear that the extent of proliferation of this tissue in the trout will prove to be as useful in quantitation of dietary aflatoxin.

The extent of nuclear aberration in the trout liver gives promise of being a useful indicator of dietary toxicity. We have divided the continuum of nuclear aberrations into three arbitrary index figures, No. 10 being a situation in which very few parenchymal nuclei show greater than normal size or aberration of shape. No. 12 is the most extensive deviation in which many nuclei are gigantic and bizarre in shape. We are now attempting to correlate the extent of nuclear deviation with the aflatoxin level. We are not prepared at this time to say that aflatoxin is the only component giving rise to nuclear aberration. From whatever cause we assume that the nuclei are prevented from dividing normally, and as a result the entire cell increases in size and eventually the nucleus starts to assume bizarre shapes. In the end, these huge, bizarre nuclei disintegrate and the space occupied by the cell is filled with secondary generation parenchyma. We have also been interested in the appearance of bizarre nuclei in mouse liver parenchyma. These are found in mice which have been fed the Santa Monica pellets (commercial ration) for 15 months. Nuclear aberrations almost as pronounced are found in the livers of mice fed Purina Mouse Chow for a

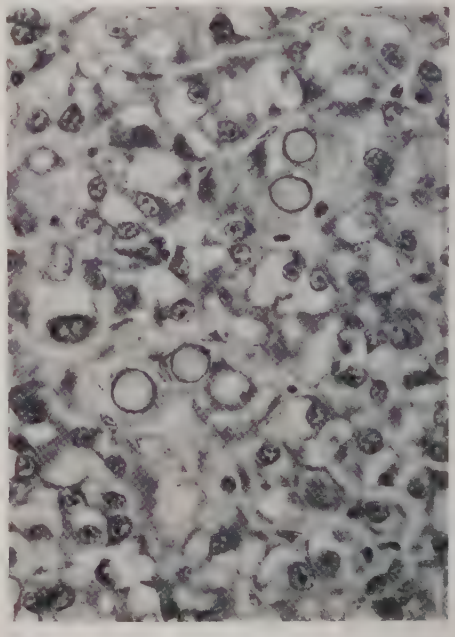

FIGURE 14.—Vacuolated nuclei are present in some degenerating liver cells. Note the cytoplasmic vacuoles originally containing glycogen. H. & E. × 320.

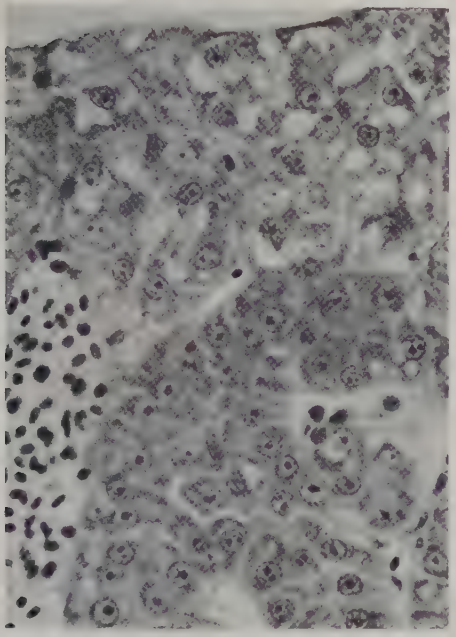

FIGURE 15.—The margin of a hepatoma node (left center) contrasts sharply with the "normal" liver tissue. Note the smaller, more basophilic cells of the tumor. The larger, eosinophilic cells of the "normal" parenchyma always contain more glycogen than do the tumor cells. H. & E. × 320.

year. The nuclei of wild, house mice show some variation in size, and we assume this to be normal, but they do not show the same degree of variation in size and shape found in either the Santa Monica or the Purina fed groups. We hope to examine this matter further.

How can we relate these observations of the liver disorders in trout to hepatoma? Is it necessary for such degenerative changes to occur before hepatoma formation is possible? Is hepatomagenesis in trout a two-step process, and if so are the changes we observe through light microscopy indications of a cellular alteration which must occur before a mutation ending in hepatoma is possible, or on the other hand are these cytological changes we observe actually unnecessary for mutagenesis? In our diet 16, which is the synthetic diet (OTD) with 4 ppb of crude aflatoxin added we can see no clearly defined cytological changes although some minimal incidence of hepatoma (2/14) was observed at 9 months. This does not mean that cell damage does not occur because the required preliminary steps may be most difficult to observe. Nevertheless it does seem probable that the gross evidences of cell damage and the very high incidence of hepatoma in several other diets are causally related. The experimental program of the coming year will be designed in such a way that we may be able to assign an order of importance to the various types of damage which precede hepatomagenesis.

A question of basic importance is whether the mutant cell which results in a tumor is of the primary or secondary generation. In the first place we feel reasonably certain that it is a parenchymal cell because of the many similarities it has to the normal parenchymal cell. The mutation might be expected to occur in a chromosome of a primary generation cell because the duration of the mitotic stages seems to be much greater than of those in the secondary generation. If the accumulation of a carcinogen is important it is obvious that such an accumulation is probable in the primary cells which appear to exist for several months before the first micronode is seen. On the other hand there is much less time for an accumulation of a mutagen in the cells of the secondary generation. Lastly we have seen tumors developing in livers where it is difficult if not impossible to demonstrate the presence of secondary generation parenchyma.

ULTRASTRUCTURAL AND BIOCHEMICAL OBSERVATIONS ON TROUT HEPATOMA

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In an earlier study [*1*] it was found that trout hepatoma is accompanied by elevations of serum proteins. In some cases a threefold increase over normal values was noted. The composition of neoplastic serum was qualitatively similar to that of normal serum except for the presence of a component with a greater mobility than albumin (table 39-A).

Histologically these tumors contain varying amounts of eosinophilic material present in extracellular deposits. Histochemical studies showed this material to be a basic protein and to contain the amino acids arginine and tyrosine. The tumor cells contained similar staining which was concentrated at the cell periphery. The tumor cells were intensely basophilic. At the level of ultrastructure the endoplasmic reticulum (ER) was highly developed and the cisternae contained amorphous material. The Golgi complex was also highly developed in neoplastic hepatocytes, it consisted of the characteristic lamellae and vesicles and was associated with large numbers of dense granules. At the cell periphery the ER appeared less organized and more free ribosomes were present. Profiles of cisternae were observed which appeared to open into the dense zones. The plasma membranes were vesiculated and in several instances dense material appeared to be passing into the extracellular space. In an effort to study amino acid incorporation, microsomes were isolated from non-neoplastic and neoplastic trout livers and incubated under appropriate conditions with C^{14} lysine. Microsomes from trout hepatoma incorporated C^{14} lysine to a greater degree than did particles from non-neoplastic livers, the highest values were obtained with microsomes isolated from a hepatoma rich in extracellular eosinophilic material (table 39-B).

TABLE 39-B—*Incorporation of C^{14}-lysine in microsomes*

	CPM/cm²
Hatchery trout	138
Wild trout	119
Adenoma	207
T. Hepatoma	113
"F" Hepatoma	392

Incubation of 3 Mg microsomes, 0.08 μM C^{14}-Lysine, 10 μM PEP, 1 μM ATP, 0.2 μM GTP: 20 min. at 35°C.

These results suggest that some trout hepatomas possess an augmented capability to synthesize plasma proteins and

TABLE 39-A

Source of serum	No. sera tested	Av. total serum protein (gm. per cent)	Percent component in serum				
			1	2	3	4	5
Non-neoplastic	11	3.6	4.0	24.7	37.7	33.3	
Adenoma	4	6.1	6.7	27.4	31.8	25.5	7.6
Hepatoma	9	9.8	4.8	26.2	37.5	24.8	6.5

may be looked upon as "functional" tumors.

REFERENCE

1. Cancer Research *23*:848–857, 1963.

Discussion

DR. SCARPELLI: I would like to preface my comments by saying, that Dr. Snieszko and his group at the Eastern Fish Disease Laboratory at Leetown, W. Va. have been extremely cooperative in supplying me with all the material for this study.

In 1963 we were struck with the finding that fish-bearing liver tumors showed elevations of serum proteins. In some of the more advanced tumors the elevations were very marked indeed, and this is what stimulated the study that I am going to report on now.

Figure 16 shows the various types of liver lesions that we encountered. These ranged from a slightly enlarged liver with many small nodules which were classified as adenomas, to a large liver which contained large confluent tumor masses which were carcinomatous. In this particular instance there were three distant metastases in the gill, spleen and heart.

Figure 17 shows the histologic pattern of most of the hepatocarcinomas we studied, these were the so-called trabecular hepatomas.

Dr. Snieszko made the first observation that trout bearing liver tumors

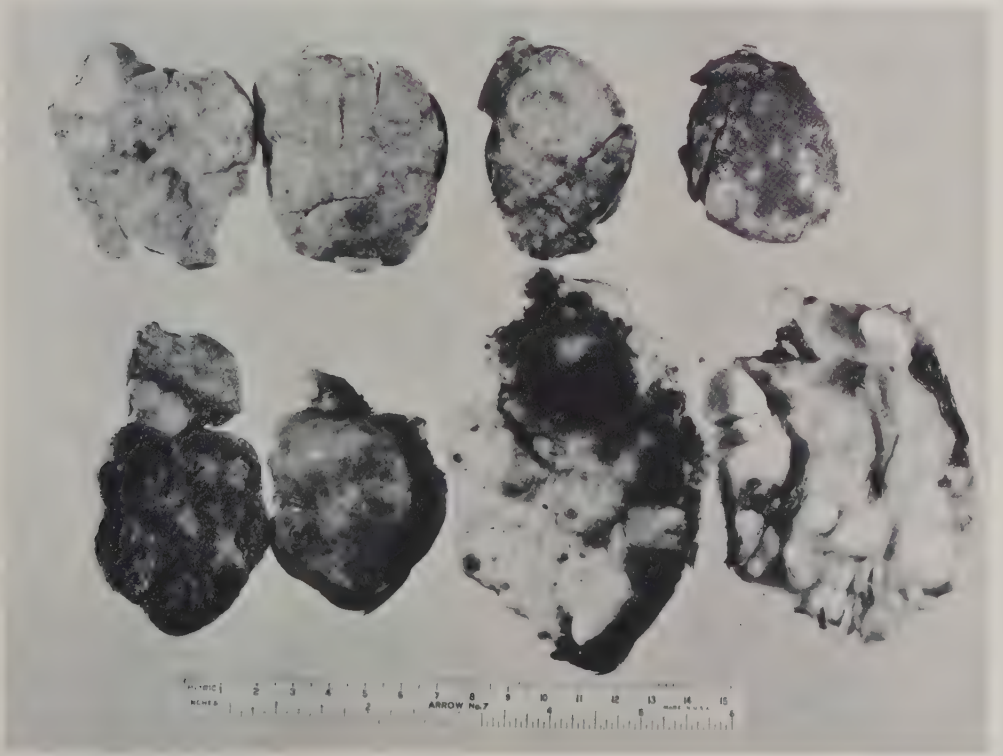


FIGURE 16.—Gross photograph of typical hatchery-reared trout liver lesions. In the larger tumors hemorrhage and necrosis were a frequent finding.

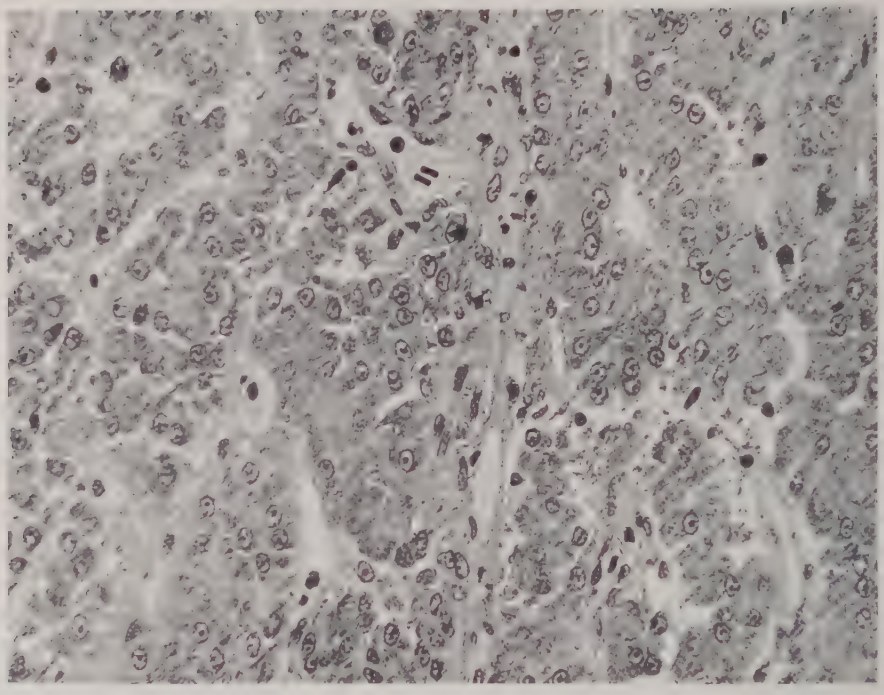


FIGURE 17.—Photomicrograph of a liver tumor showing the most commonly encountered histologic pattern, that of a trabecular hepatoma in which cords of tumor cells are arranged in such an abnormal fashion that normal tubulosinusoidal structure is disturbed. Magnification × 490.

showed marked elevations of their serum proteins. Electrophoretic patterns of normal trout serum and tumor bearing trout serum are shown in figure 18.

In table 39-A of the manuscript, it can be seen that in trout with hepatic carcinoma the total serum proteins may be elevated three-fold. Furthermore, the elevation was reflected by an increase of all of the serum protein components. Component 5 is present in only adenoma and hepatoma and migrates more slowly than albumin, its significance is still obscure.

When one studies the histology of these tumors, it is quite clear that cytoplasm of the tumor cells is basophilic. Figure 19 shows a tumor nodule in liver. The nodule is deeply stained by the dye Azure B which at an acid pH specifically stains ribonucleic acid (RNA). If one pre-treats this tissue with RNA-ase, an enzyme capable of degrading RNA, one finds that the staining is completely abolished.

This nodule represents one of those bothersome nodules which defy unequivocal diagnosis. In the absence of wide spread intrahepatic involvement and more importantly, extrahepatic extension or metastases, we considered it nonmalignant.

Hatchery-reared trout liver cells not uncommonly have a vacuolated appearance, which was interpreted by some as being indicative of cytoplasmic lipid. When a PAS reaction is performed it appears that much, if not all, of the vacuolated material is glycogen as is shown in figure 20.

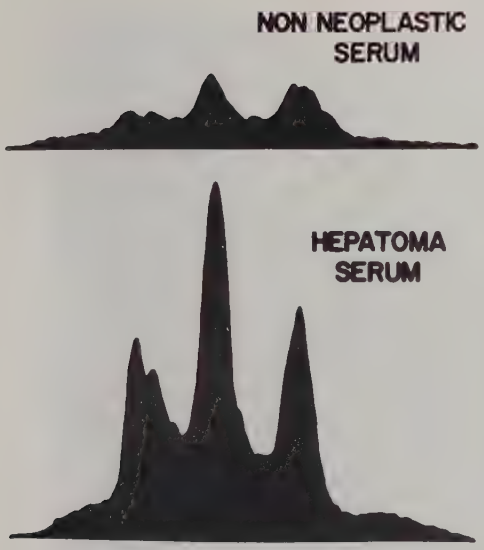


FIGURE 18.—Electrophoretic patterns of serum from normal and tumor-bearing trout. Marked elevation of all plasma protein components serum from the fish with hepatoma is clearly evident.

I would like to draw your attention to an electron micrograph (fig. 21) of a 3-year-old hatchery reared rainbow trout. Here one finds endoplasmic reticulum which consists of highly organized arrays of membranes associated with small granules called ribosomes. This component of the cell is where protein is synthesized.

Now, when one looks at hepatoma cells (fig. 22), one sees that the endoplasmic reticulum is even more highly organized. Notice that in the extracellular space between tumor cells there are some accumulations of electron-opaque material.

In about 20 percent of the hepatic tumors we found that the extracellular space was filled with amorphous palely eosinophilic material (fig. 23). This has been proposed by some as probably indicating a degenerative change, but looking at this a little more carefully, one becomes impressed by the fact that these cells do not look necrotic. Furthermore, this material was consistently present in tumors from animals that showed very high elevations of their plasma protein level.

When we stained this material with fast green at pH 2, which stains basic proteins, it is possible to demonstrate that this material between the cells is indeed a protein (fig. 24), and other tests have shown it contains both the amino acids lysine and arginine.

Electron microscopy of tumors from animals with the highest elevations of their plasma proteins revealed cisternae of the endoplasmic reticulum which were filled with an amorphous fluffy appearing material, with occasional dense granules that closely resembled secretion granules (fig. 25). At the plasma membrane one often encounters vesicles which contain dense material and some of which appear to be emptying their contents in the extracellular space.

In figure 26 one can see this amorphous material at a higher magnification, also the bizarre configurations of the plasma membrane at the cell surface.

This is a preliminary piece of work which was done in an attempt to see whether or not the microsomes isolated from non-neoplastic trout livers had a different rate of incorporation of C–14 labeled lysine than those of neoplastic livers. It was found that microsomes isolated from trout liver tumor cells incorporated C–14 labeled lysine more rapidly than microsomes from non-neoplastic liver cells. These experiments were done at 35 degrees centigrade, not 23 degrees centigrade, which appears to be optimal for this system so that these experiments must be repeated.

In an effort to ascertain whether we could find any biochemical differences between non-neoplastic livers, adeno-

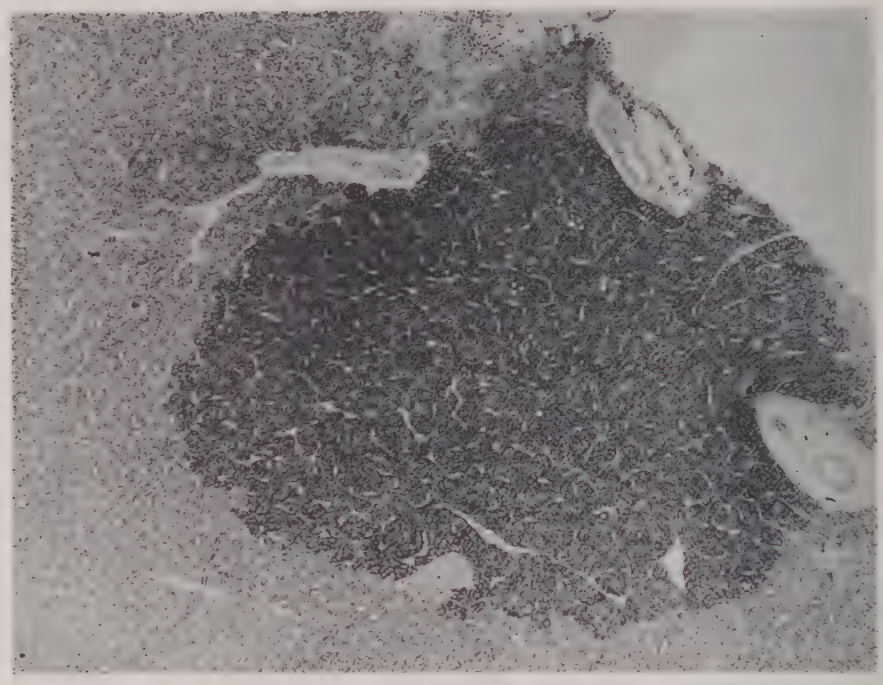


FIGURE 19.—Basophilic nodule stained with Azure B to demonstrate an increased amount of cytoplasmic ribonucleic acid. The surrounding normal liver cells show a minimum of cytoplasmic basophilia. Also note that the muralia or cords are thicker in the basophilic nodule than in the surrounding liver. × 110.

mas, and carcinomas, various enzyme activities were studied. The enzymes we studied were transaminase, succinic, lactic, isocitric and glucose-6-phosphate dehydrogenases, and aldolase and leucine-aminopeptidase. We could find no biochemical differences between so-called adenomas and frank hepatocarcinomas.

A series of phosphatase enzymes which included ATP, both magnesium and calcium-activated, alkaline phosphatase, acid phosphatase, and glucose-6-phosphatase were also of no value in differentiating between adenomas and hepatocarcinomas.

In a single tumor there were large amounts of lipid in the tumor cells (fig. 27). Since these cells did not show evidence of injury at the level of ultrastructure (fig. 28) this could be interpreted as evidence of excessive mobilization of depot fat, or a failure of export of fat from the liver, or finally, augmented synthesis of fat by neoplastic hepatocytes.

Spontaneous trout hepatoma may be an ideal model for studying so-called functional tumors. These are neoplasms that characteristically are slow growing, metastasize late and rarely, and retain their capacity to function in the biochemical sense at least, as liver cells. In some cases the function is greater than normal; the precise significance of this augmentation and its relation to the growth potential of these tumors remain to be clarified and merit further study.

From a comparative point of view, it

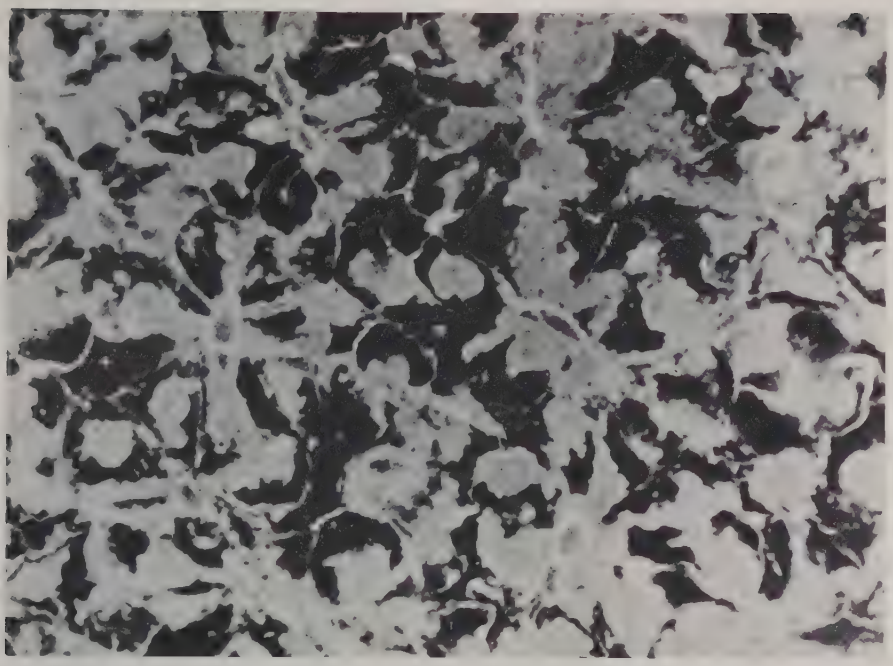


FIGURE 20.—Periodic acid Schiff (PAS) reaction in the normal liver of a 3-year-old trout showing massive accumulation of glycogen in the liver cells. The faint outlines of nuclei are evident. × 1,200.

is of interest that in man also a variety of metabolic disturbances have been observed in association with hepatic carcinoma; these include sequestration of glycogen by the liver with hypoglycemia, hyperalbuminemia, hyperglobulinemia, and increased hepatic lipid.

DR. LA ROCHE: I have heard that liver vacuoles are all glycogen containing. I have done a good amount of histology in trout and salmon and found with Sudan Black the indication that some of those vacuoles at some time are mostly fat. Have you ever considered that at certain times of the year or at certain stages in development one can find as much as 20 percent fat in the liver, so it has to be someplace?

DR. SCARPELLI: The material I have had access to has been from hatcheries, and I have uniformly found that the amount of lipid that is stained by either Sudan Black or any of the Sudan stains, Sudan Red, for that matter, is very small. In fact, most of it appears to be ceroid which is distributed around intrahepatic bile ducts.

DR. LA ROCHE: How do you define "ceroid"?

DR. SCARPELLI: By "ceroid" I mean oxidized fats, very high molecular weight, lipid peroxides. These are referred to as ceroid. These are insoluble fat complexes which are present. This is not neutral lipid because it withstands alcohol dehydration, and to my knowledge, true neutral fat disappears in the alcohol dehydration step used in the routine H & E stain.

When one does an oil red stain on a frozen section a few scattered droplets of neutral fat are seen. The bulk of the

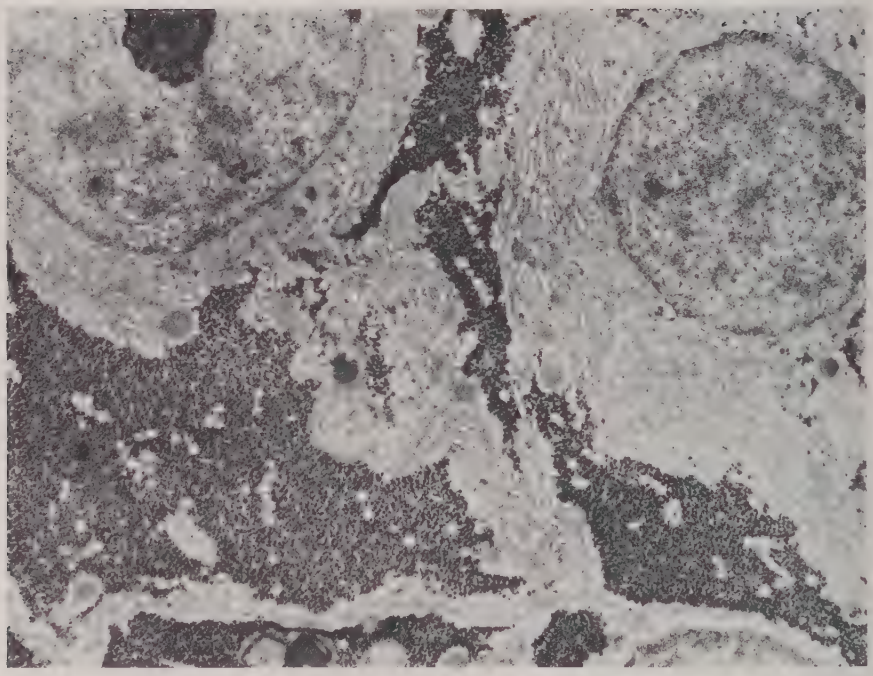


FIGURE 21.—An electron micrograph of the liver shown in figure 20. The dense granules represent deposits of glycogen, note that this occupies a large percentage of the cytoplasmic volume. The parallel arrays of endoplasmic reticulum are also evident. × 7,300.

cytoplasmic vacuolization appears to be glycogen.

DR. LA ROCHE: However, sometime in the development of the fish the liver fat has to be some place. This fat contains cholesterol, triglycerides, and phospholipides. One can find from 3 to 20 percent total fat in the liver, and a visual difference may reside in the paler appearance of the liver containing the most fat. I have seen quite a bit of livers that were staining beautifully with the vacuoles inside the cells, staining with Sudan Black. Liver lipid content may be greatly influenced by qualitative and quantitative intakes. I just want to emphasize the fact that it isn't always all glycogen. It could be fat, at least partially, under some conditions.

DR. SCARPELLI: I am sure fat can occur. Ghittino has shown much fat in the Italian trout, but in the trout I get from West Virginia there is very little fat in the livers, at least under the conditions I have looked at. Most of it is glycogen.

DR. HALVER: Many of the fish that you used originally, Dr. Scarpelli, were rather old fish, rather large, and they did have a rather high glycogen content from close visual observations and a review of their dietary history. In contrast young salmon and trout do often have up to 20 percent lipid in the liver and the liver appears normal. Now, these vacuoles you have seen and have stained and identified as glycogen were not the type that will stain with Sudan Black.

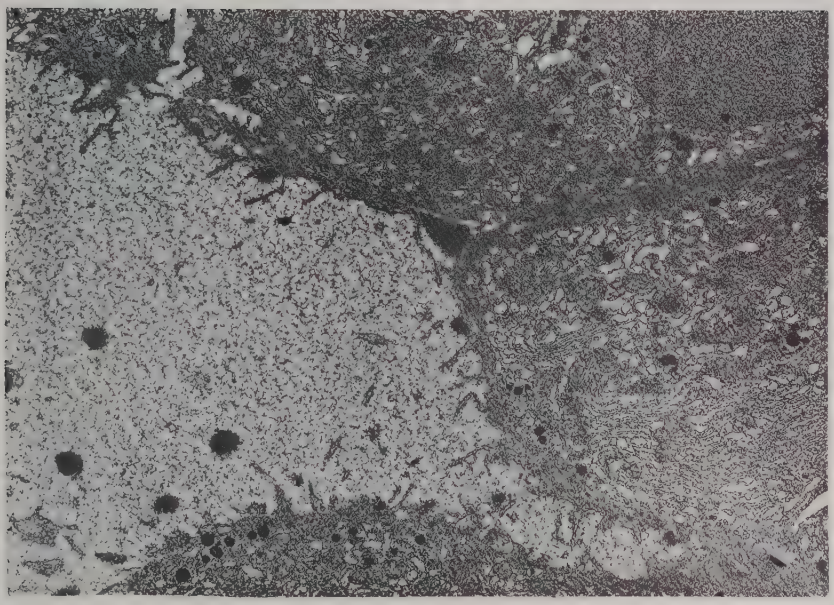


FIGURE 22.—An electron micrograph of a sinusoidal space in a hepatoma. The sinusoid is bordered by three tumor cells. Compare the absence of glycogen and the extensive development of the endoplasmic reticulum. Note the dense and fluffy material in the endoplasmic reticulum, at the cell borders and in the sinusoidal space. × 5,800.

DR. SCARPELLI: Fat vacuoles have a much sharper edge than glycogen vacuoles. Glycogen has a foamy, fine, verticulated appearance, whereas neutral fat has a very different appearance.

DR. HALVER: When one feeds trout high levels of carbohydrates in confined environment one almost always ends up with high glycogen livers. Other abnormalities in the diet, improper vitamin balance and so forth, will also contribute to this condition. When one feeds high quantities of digestible fat one ends up with a fatty liver. There are two or three different aspects to consider here.

DR. NEWBERNE: I would just like to ask, at what point during the course of tumor induction can you detect the increase in serum protein? How long does it take before you can find it?

DR. SCARPELLI: The study I have reported on deals wholly with spontaneous hepatomas.

DR. SIMMONS: You described these ceroid pigments as being peroxides and polymerized fat. Could you be more definite on the description of "ceroid"?

DR. SCARPELLI: Ceroid is a lipid pigment which it is generally considered arises from the autoxidation of phospholipid and unsaturated fats. This oxidation is accompanied by polymerization so that the final product is highly insoluble even in organic solvents. This material is characteristically acidfast and histochemical studies have shown that it contains peroxide groups. I am referring to the Dam re-

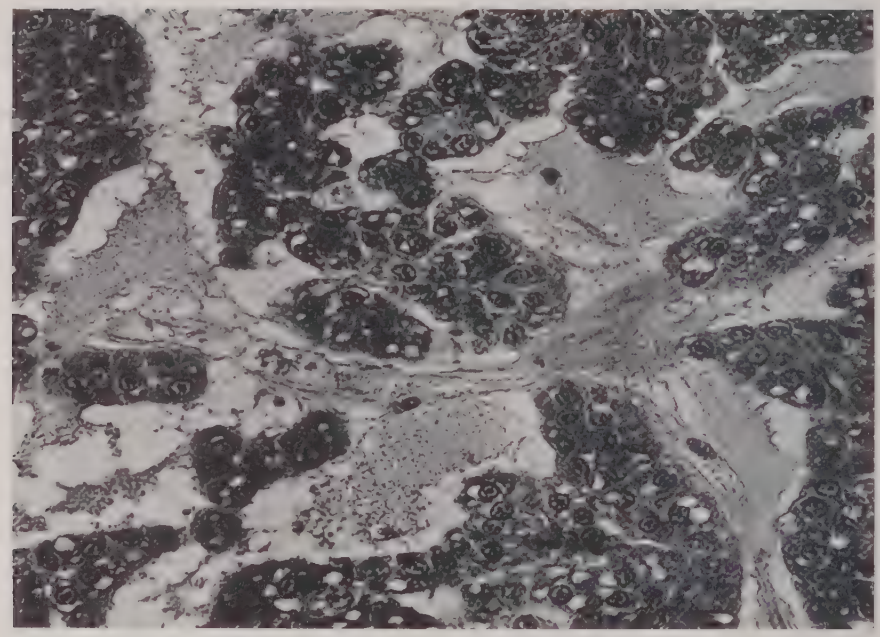


FIGURE 23.—A histologic section showing the accumulation of proteinaceous material in dilated sinusoidal spaces. Note the well preserved tumor cells and the absence of foci of necrosis. × 490.

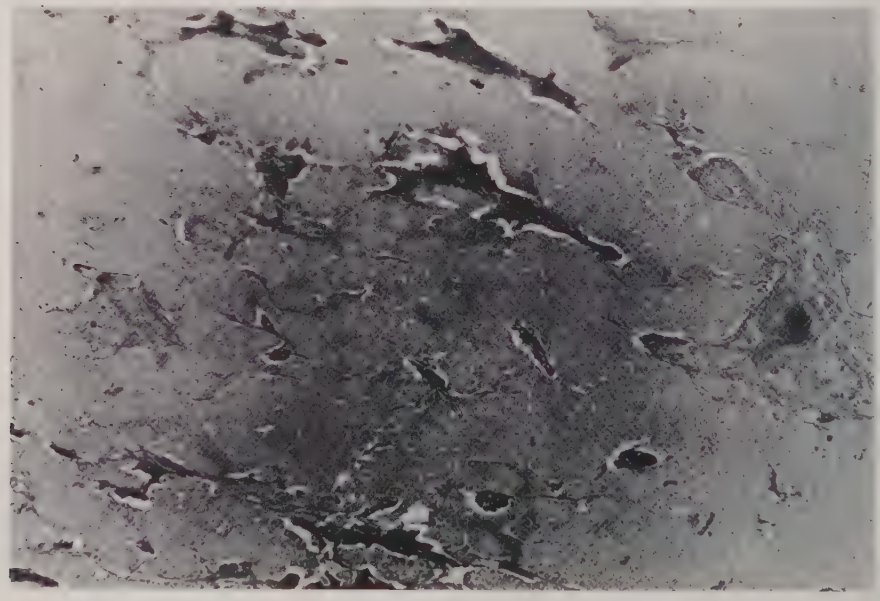


FIGURE 24.—A section of hepatoma stained with fast green at pH 2 showing that the dense material in the sinusoids is a basic protein. × 60.

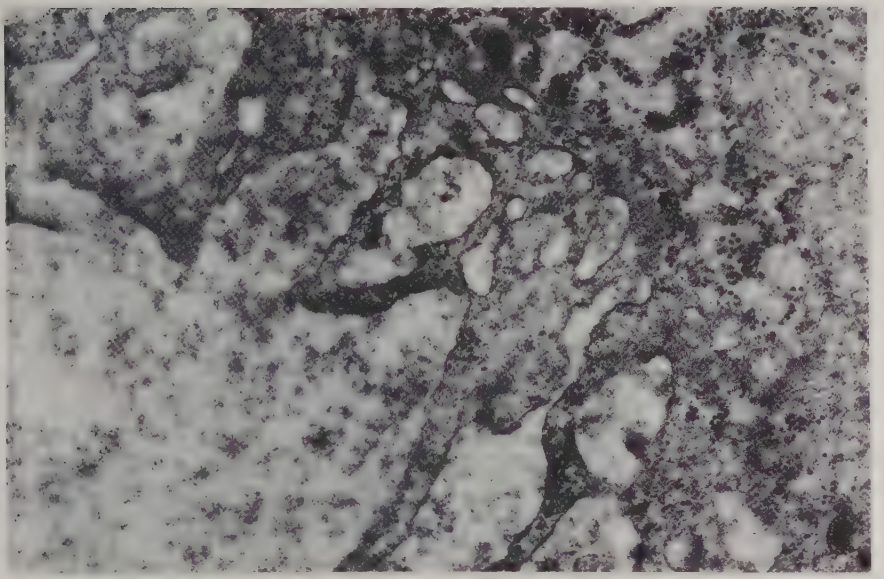


FIGURE 25.—An electron micrograph of the plasma membrane of a neoplastic liver cell lining a sinusoid. Note the extensive infoldings of the plasma membrane and numerous small vesicles. Amorphous material may be seen at the cell border and in the sinusoid which is shown in the lower left hand corner of the photograph. × 63,000.

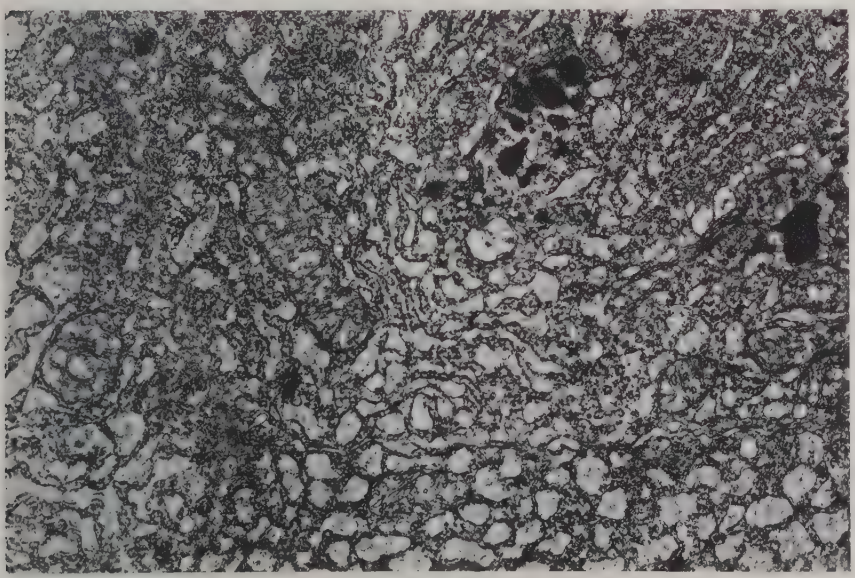


FIGURE 26.—An electron micrograph of the cytoplasm of a neoplastic liver cell. The endoplasmic reticulum is highly developed and contains many dilated cisternae filled with fluffy amorphous material. Note also the presence of dense granules which do not have the characteristic appearance of lipid. × 13,000.

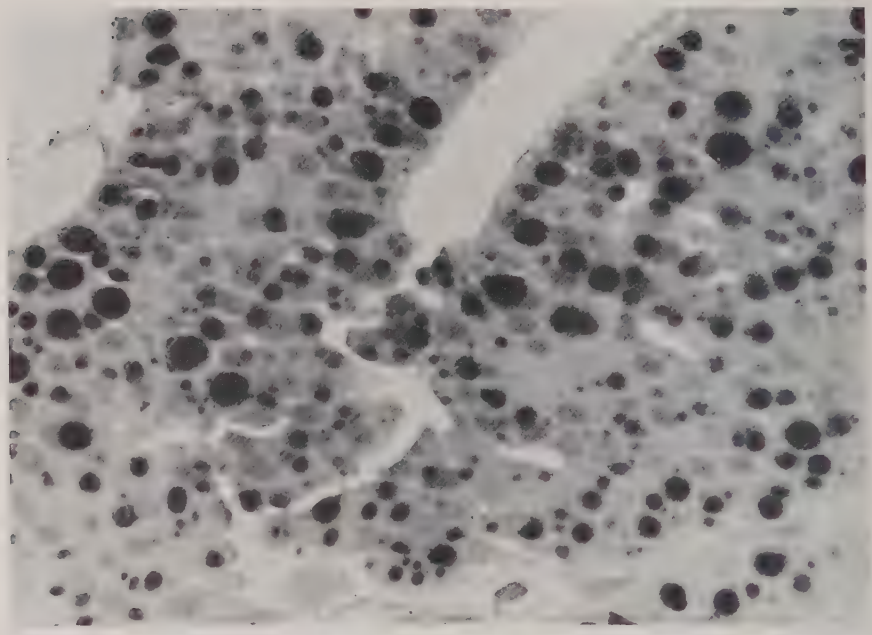


FIGURE 27.—A section of hepatoma stained with osmium tetroxide. The large black droplets consist of lipid. × 700.

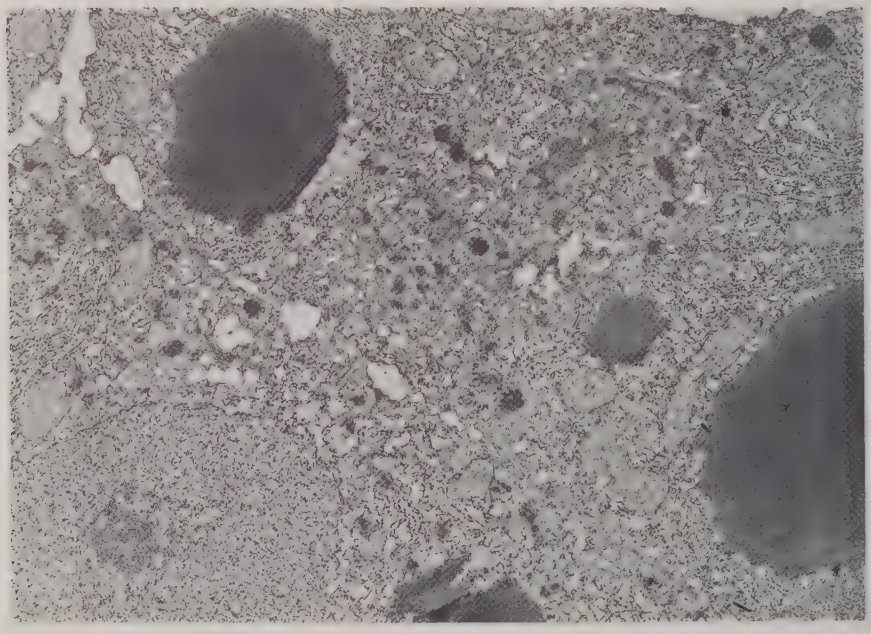
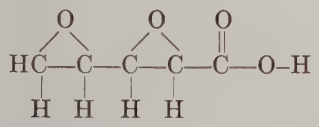


FIGURE 28.—An electron micrograph of the liver shown in figure 27, showing well preserved malignant cells. There is no evidence of acute cell injury. × 8,500.

action for lipid peroxides. The general structure of lipid peroxides would be as follows:



An excellent reference on the chemistry of ceroid is that found in the second edition of Pearse's treatise on Histochemistry, pages 661–668, Little, Brown and Company, Boston, 1960.

DR. KUMMEROW: Ceroid is a product of fat oxidation *in vivo*, but it does not seem to contain peroxide. The peroxide can be found because it is very difficult to isolate highly unsaturated lipids without peroxidation *in vitro*. To date, peroxides have not been demonstrated to occur *in vivo*, although "ceroid" can be formed by a combination of proteins with aldehydes and ketones, which are products of fat oxidation.

AMINO ACID INCORPORATION IN RAT AND TROUT LIVER

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Dr. Scarpelli has discussed briefly some studies on the *in vitro* incorporation of amino acids by microsomes from normal trout liver as compared with microsomes from hepatoma and has found the incorporation by the preparations from the hepatoma to be significantly greater. Mr. Ed Murray in my laboratory has been studying amino acid incorporation by microsomes and ribosomes from normal rainbow trout liver and I would like to show you some of the data he has obtained.

But before showing these data I would like to discuss some findings we have made with the regenerating rat liver. During the past 3 years Drs. Cammarano and Melli and I have been interested in trying to understand the basic mechanisms that might be involved in carcinogenesis. We decided to use the regenerating rat liver as a model system to assess what changes are induced by extirpation of 70 percent of the liver that results in the increased rate of protein synthesis.

As most of you know, the parenchymal cells of an adult liver do not divide very often. I think it has been estimated that they divide about once a year [*1*]. On the other hand, the liver itself will produce an amount of protein equal to its own weight in about 6 days, so that it is largely a manufacturer of protein, and most of this protein is for export purposes.

Now, if one surgically removes 65 to 70 percent of the liver, then a whole series of changes are initiated that culminate, at about 24 hours, with all of the cells in the residual stump dividing synchronously, and they will go through two or three of these synchronous divisions. The surgery has triggered a whole new set of processes in this liver, and these continue until the liver is almost fully repaired and then the process slows down and the liver returns to its normal job of making export proteins.

A series of new enzymes are induced by the surgery, largely enzymes involved in DNA synthesis to sustain the mitoses that are subsequently going to occur. It is also well known that protein synthesis *in vivo* in a regenerating liver is increased.

We studied the biochemical changes during liver regeneration very carefully from a lot of standpoints but I will just summarize the distinct findings that we made.

We first made an examination of the ribosomes or the attachment of the ribosomes to the endoplasmic reticulum. Operationally this is done by taking a post mitochondrial supernatant and centrifuging it through a discontinuous gradient of a halfmolar sucrose overlaid over a 2 molar sucrose phase. What happens is that the endoplasmic reticulum, being high in phospho lipids, will not penetrate the 2 molar sucrose. However, the ribosomes being fairly dense,

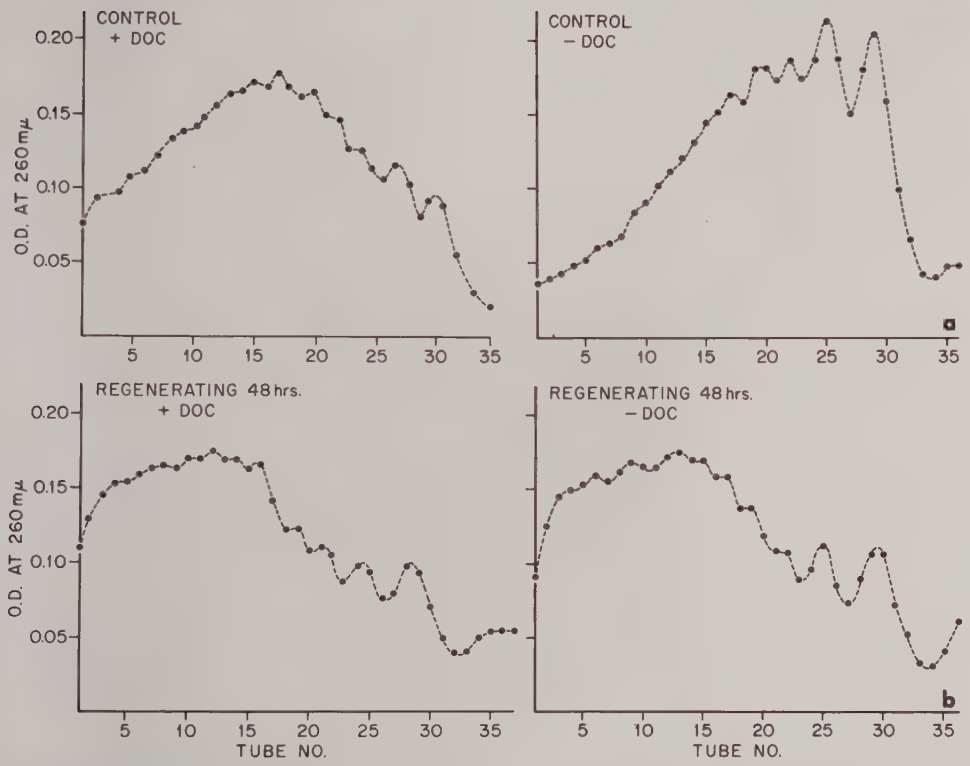

FIGURE 29.—Sucrose density gradient patterns of C ribosomes obtained in the presence (+DOC) and absence (—DOC) of deoxycholate. The C ribosomes were layered on top of 28 ml linear (10–34 percent) sucrose density gradients in Sw 25 tubes. The tubes were centrifuged at 25,000 rpm in the Sw 25 rotor for 120 min. After centrifugation the tubes were punctured, drops were collected, and the optical density at 260 mμ was measured.

if they are lying free, will sediment through the 2 molar sucrose as a pellet and then one can examine these in a sucrose density gradient.

Webb and Potter [*2*] had shown that there are some quite distinct differences between certain hepatomas and normal liver in this regard, therefore we made a similar study during liver regeneration.

If one treats the post mitochondrial supernatant with deoxycholate (DOC) which dissolves the membranes, then one will get the total particles. Then by comparing the particle distribution in a sample that was treated with deoxycholate and one that was not, we could ascertain something about the attachment of the particles to the membrane.

Figure 29 shows the distribution of monoribosomes and polyribosomes from post mitochondrial supernatants from normal livers and livers that have been regenerating for 48 hours. On the right hand frames are particles obtained in the absence of deoxycholate. The point of interest here is that in a normal liver very few polysomes are found if the preparation is not treated with deoxycholate. Such treatment results in a large increase in polysomes by dissolution of the membranes. With the re-

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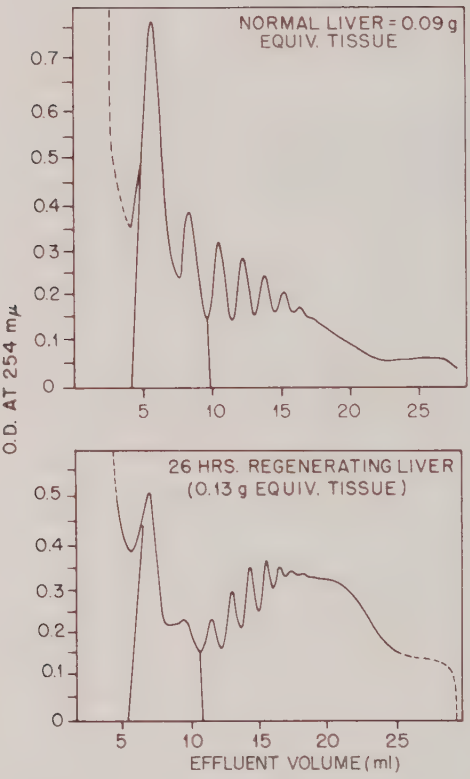

FIGURE 30.—Ribosome distribution pattern of normal and regenerating liver. Livers were homogenized with 3 volumes of medium A and the post mitochondrial supernatant (PMS) was collected, adjusted to 1.2 percent DOC and layered as an inverted sucrose gradient on top of 28 ml of a convex exponential (10–24 percent) sucrose gradient. Centrifugation as in figure 29.

generating preparations however there is no difference in the yield of polysomes whether or not the preparation is treated with DOC. It almost seems that during liver regeneration the polysomes are not attached to the membranes but sediment as if they are free in the cytoplasm. I can assure you that in this case these polysomes are indeed still bound to the endoplasmic reticulum, but there has been a reorganization of the nature of the binding so that they can be removed by the centrifugal force applied during this procedure.

The other thing that we found in regenerating liver was an alteration in the size of the monomer peak with respect to the polysomal region as compared to normal, and this is shown in figure 30.

In this case we have a gradient going in the opposite direction.

The monomer peak is at the left and they increase in size from left to right. The upper part of the figure shows the relative distribution of the monoribosomes to the polyribosomes in a normal liver and the low tracing shows the situation in a liver that has been regenerating for 24 hours. The striking point is that during liver regeneration there is a sharp decrease in the size of the monomer pool and a corresponding increase in the polysomal region. We have shown previously that the monomers are not engaged in protein synthesis *in vivo*—only the polysomes are active. Therefore it seems logical that an increase in the rate of protein synthesis can be accomplished by increasing the number of polysomes at the expense of the monomers. Indeed by planimetry we calculated that during liver regeneration there is a 25 percent increase in the area of the polysomes and this corresponds to the 25 percent increase in the rate of protein synthesis that one observes during liver regeneration.

We then went on to conduct some *in vitro* amino acid incorporation experiments with microsomes and ribosomes prepared from rat liver at varying times during liver regeneration. In order to avoid any complications that might be introduced by differences in the rate of messenger RNA synthesis at different times during regeneration we used a synthetic messenger, polyuridylic acid (poly U) that catalyzes the synthesis of polyphenylalanine. We

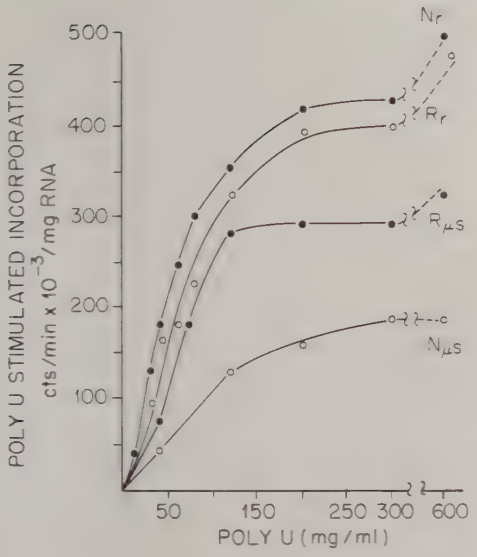

FIGURE 31.—Amino acid incorporation by microsomes and ribosomes from normal and regenerating livers. Each incubation mixture included (per ml) Tris-HCl pH 7.6 50 μ moles; Mg acetate 8 μ moles; creatine phosphate 12.5 μ moles; creatine phospho kinase 20 μ grams; ATP 0.5 μ moles; GTP 0.5 μ moles; U—C^{14} phenylalanine (SA 360 μ C/μ mole 1×10^6 cpm); microsomes (μs) or ribosomes (r) 300 μ grams of RNA, supernatant fraction 3 mg of protein.

compared the ability of microsomes and ribosomes from normal and regenerating liver to catalyze the poly U dependent incorporation of phenylalanine. Representative data are shown in figure 31. These preparations were all tested with a common supernatant, so we are measuring only the differences in the particulate fractions. It is quite evident that microsomes from regenerating liver ($R_{\mu s}$) are about twice as active as microsomes from regenerating liver ($N_{\mu s}$). However, when these microsomes are treated with deoxycholate to convert them to ribosomes, the difference between the preparations from normal and regenerating livers disappears. There appears to be something associated with the membrane that is dissolved by DOC that regulates the rate of the incorporation process. We made a quite careful study of the poly U stimulated incorporation as a function of time after surgery and these data are summarized in figure 32.

These are all *in vitro* incorporation experiments with microsomes isolated at various times after surgery. The curve labeled homologous means that at the points indicated the microsomes were tested with a supernatant isolated at that same time. The curve labeled regenerating means that the microsomes isolated at the times indicated were tested with a single common supernatant isolated from a liver that had been regenerating for 52 hours. The curve indicated as normal refers to microsomes isolated at the times indicated, but tested with a supernatant isolated from a normal liver. The lower dotted curve represents data obtained with microsomes isolated from a nor-

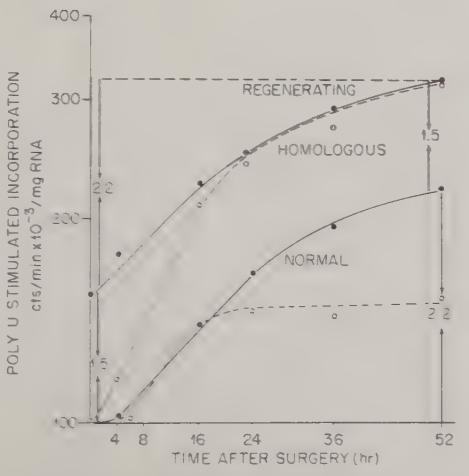

FIGURE 32.—Poly U stimulated incorporation of phenylalanine as a function of time after surgery. Experimental conditions as in figure 31.

mal liver, but tested with supernatants isolated at the indicated times after surgery. This shows that normal microsomes are increasingly stimulated by the supernatant from regenerating liver up to about 18 hours after surgery. Thereafter the supernatants are all the same. The homologous curve indicates two components, one at the level of the microsomes and one at the level of the supernatant. This is more evident in the curve for the heterologous experiment. Here the microsomes were all tested with a 52-hour supernatant. The rising activity in this case is all attributable to an increasing responsiveness of the microsomes. All of these data taken together suggest that during liver regeneration changes are taking place at the level of the endoplasmic reticulum that involve some sort of reorganization so that the binding factors have been altered. There seems to be a component that is easily dissociable from the membranes, or at least soluble in DOC, which regulates the rate at which ribosomes can interact with messenger RNA.

I would like now to talk for just a few minutes about trout liver. Our overall plan is to look for changes in trout liver during carcinogenesis similar to those we have observed in regenerating rat liver. When we started to work with the livers of rainbow trout we found that except for a paper by Dr. Scarpelli [*3*] there were few data in the literature on the biochemical aspects of metabolism or protein and nucleic acid synthesis in trout liver, or for fish liver in general. We therefore worked with normal trout liver to establish some basic parameters upon which to gauge any changes that might be seen in a hepatomatous liver.

We first studied the *in vitro* incorporation of amino acids with microsome preparations from rat liver and other tissues and all of these reactions were carried out at 37° C. Therefore we carried out the experiments with the trout preparations at this temperature. When we compared the extent of incorporation of C^{14} phenylalanine by the preparations from trout liver with those from other tissues, the trout liver preparations seemed comparatively quite inefficient. So we measured the effect of temperature on the incorporation process. Representative data are shown in figure 33. It is clearly evident that a temperature of 23° C. is optimal for the fish system. This however is not the correct temperature for these fish, I doubt if they would live very long at this temperature. We will have to study this effect a little more in order to understand why there should not be a lower optimal temperature.

In an effort to determine why the *in vitro* system incorporates for only 5 minutes at 37° we decided to cross components with the rat system that has an optimal temperature at 37° C. We used ribosomes from trout liver with supernatant from rat liver and the reverse combination, ribosomes from rat

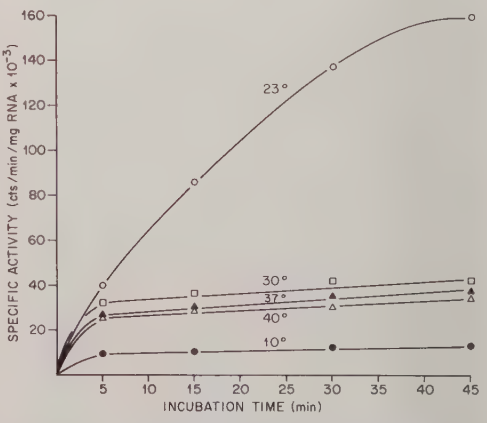

FIGURE 33.—In vitro amino acid incorporation by trout liver preparations; effect of temperature.

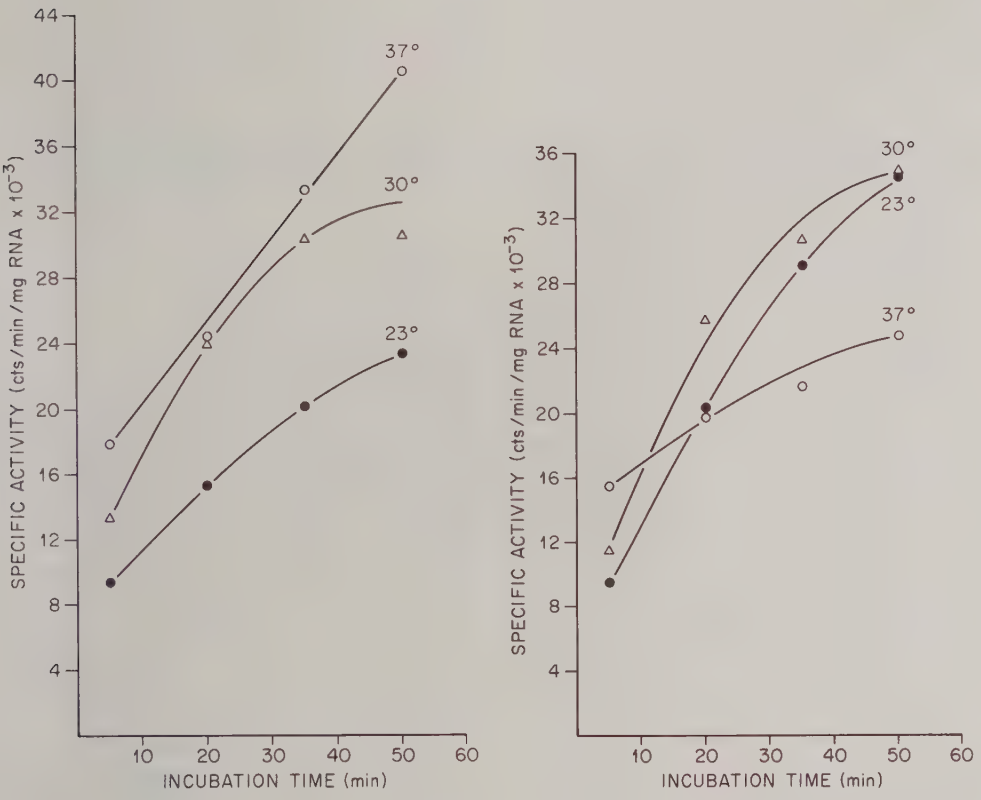

FIGURE 34 (A and B).—Amino acid incorporation by mixed rat and trout liver preparations. Left—data from experiments with ribosomes from trout liver and supernatant fractions from rat liver. Right—data from experiments with ribosomes from rat liver and supernatant fractions from trout liver.

liver and supernatant from trout liver. These data are shown in figure 34. It is quite evident that the source of the supernatant determines the temperature optimum. That is rat ribosomes with trout supernatant incorporate best at the lower temperature, whereas trout ribosomes with rat supernatant incorporate best at the more elevated temperature. We next studied the partial reactions and found that one or more of the transfer factors in trout liver supernatant is very labile at elevated temperatures.

At the present time we are studying the *in vivo* rates of synthesis of protein and nucleic acids in normal trout liver and hope soon to examine these rates in hepatomatous liver and the hepatomas themselves.

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CRYSTALLINE AFLATOXIN AND OTHER VECTORS FOR TROUT HEPATOMA

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On June 23, 1960, during a visit to Hagerman National Fish Hatchery, five 4-year-old rainbow trout (*Salmo gairdneri*) were examined, and well-developed, multinodular livers were observed in three of the five inspected. These were later confirmed histopathologically to be primary liver cell carcinoma (Wood and Larson, 1961). This became our first official notice that hepatoma was a problem in the National Fish Hatcheries. Isolated reports, however, had indicated an occasional nodular liver in widely scattered National Fish Hatcheries, State Fish Hatcheries, and private trout farms in this country (Nigrelli and Jakowska, 1955, 1961; Wales, 1964; Rucker, Yasutake, and Wolf, 1961), in different countries in Europe (Haddow and Blake, 1933; Scolari, 1953; Cudkowitz and Scolari, 1955; Leviditi et al., 1960), and in the Orient (Honma and Shirai, 1959). During the summer of 1960, intensive surveys were conducted at all National Fish Hatcheries holding large rainbow trout, and a high incidence of tumor-bearing fish was observed in those lots of fish fed for extended periods of time on dry rations (Ashley and Halver, 1961; Hueper and Payne, 1961; Rucker, Yasutake, and Wolf, 1961). The dietary of these groups of fishes was varied and indefinite with records available indicating a multiplicity of feeds because of the various purchasing policies of the different stations included in the survey.

That summer at the Fifth International Congress of Nutrition, Professor Elaine Le Breton of the Gustav Roussy Institute for Cellular Research in Villejuif, France, reported a very similar condition had occurred in some experimental rat colonies fed in their laboratories on a new commercial rat feed (Le Breton and Boy, 1960), whereas parts of the same colony continued on the former laboratory ration had not developed liver carcinoma. A subsequent research conference disclosed two probable common denominators in fish and rodent feeds as possible vectors for the appearance of liver tumors. These were fish meal and oil seed meal which by their nature widely vary in methods of production and sources of supply, in milling processes and storage conditions endured between field production and ultimate feed use. A survey of feeding records available and extensive literature review on hepatomagenesis led to research experiments designed to investigate 13 classes of potential chemical carcinogens for fish, most of which were known to induce a high incidence of hepatoma or renal neoplasms in mammals when included in the dietary. Concurrently a study of impaired lipid metabolism as a primary or secondary precursor for the appearance of rainbow trout hepatoma was

initiated in cooperation with the National Cancer Institute. Feeding trials already underway with the classical chemical carcinogens were extended to include lipid fractions from a suspect commercial ration, which occasionally was indicated to be, or to contain, a positive vector for rainbow trout hepatoma formation. Most of the chemical carcinogens used were lipid soluble, and reports showed lipid metabolism problems often occurred in fish husbandry (Wood et al., 1957). It was decided to emphasize this particular area for study in efforts to elucidate the particular factor or factors involved in rainbow trout hepatomagenesis.

The incidence of hepatoma and the relative rates of development of each particular nodule with time and under different nutritional treatments was measured by large-scale surgical inspections conducted on representative samples of various-age rainbow trout exposed to different dietaries and held at the Hagerman Nutrition Bioassay Laboratory. In late October 1960, the first large-scale detailed inspection of individual livers from 2- and 3-year-old rainbow trout was completed. Subsequent inspections on the same animals were scheduled every 3 months following the administration of nutritional and pharmaceutical treatments. In the initial studies a tumor regression diet consisting of high protein, low carbohydrate, low protected fat, high riboflavin, high alpha-tocopherol, high choline, high niacin, and high methionine was compared with the laboratory standard 50 percent of protein complete test diet (Halver, 1957; Halver, Johnson, and Ashley, 1962), with and without various corticosteroid hormones administered to subgroups on each nutritional treatment. Cortisone acetate and hydrocortisone were both tested on the basis of the work of Boy and Chany (1960), who reported that these particular compounds did induce a reversal of hepatoma in rats when these rats were on a recovery diet, provided that the hepatoma had not advanced to an irreversible point and that sufficient normal parenchymal cell tissue was still present for regeneration. These initial regression experiments with rainbow trout were not successful. However, they were only introductory in nature, were conducted with small numbers of fish, and the fish used were 3-year-old rainbow trout which had advance hepatoma nodules in the livers. Histopathological analysis of these nodules in trout after 6 months of treatment did not indicate any conclusive degree of reversal or regression of the tumors.

Since surgical techniques had been developed for other studies with fish (Robertson, 1958), this technique was adapted for use in all experiments where small numbers of fish were involved, and where individual development of the nodule needed to be observed at periodic intervals during different treatments. It was therefore simple to conduct some explant, implant, and transplant studies. Fortunately, type sera were available from previous serological studies with large rainbow trout at Hagerman, and it was possible to cross-match and type recipients with donors using three types of previously developed reagents. These simple serological techniques developed by Dr. Ridgway and his group (1962) were used in matching as closely as possible donors carrying small classical hepatomatous nodules with recipients having no visible nodule on the liver, derived from stocks of fish with a very low incidence of hepatoma.

All experimental fish were maintained on the standard complete test

diet which had remained historically low for hepatoma incidence. Donors and recipients were opened on parallel surgical tables, small hepatoma nodules, approximately 3 to 5 mm in diameter, were carefully resectioned from the liver of the donor, were each finely minced in Ringer's solution or in isotonic saline, and a small fragment, 0.5 mm^3, of hepatomatous tissue was carefully inserted through a small incision into the recipient liver, stomach, spleen, pyloric caecal area, testes, ovaries, cardiac chamber, eye, muscle, and subcutaneous fat. After 90 days, each recipient tissue was carefully resectioned, and preserved for subsequent histopathological analysis. Hepatoma tissue was discovered growing in approximately 10 percent of the recipients when the hepatoma had been transferred to the liver, to the musculature of the stomach, and to the caecal areas. Smaller numbers were successfully transferred to the ovaries, testes, cardiac chamber, or subcutaneous fat, or were found unattached in the peritoneal cavity. Only one successful transplant was detected, however, in those fish which had *not* been serologically matched between donor and recipient. Sometimes upon transfer to the liver an invasion into the musculature adjacent to the tumor was observed. In each case the transplanted tissue was confirmed histopathologically to be liver cell carcinoma. In other stocks of large rainbow trout, metastasis to other tissues did occasionally occur spontaneously, and in many very large trout multiple organ involvement was a common occurrence (Ashley and Halver, 1963).

The 13 chemical carcinogens used in the first induction study were fed to initial feeding rainbow trout fry at five levels over a 256-fold range at $\frac{1}{16}$, $\frac{1}{4}$, 1, 4, and 16 times that dosage known to induce a high incidence of hepatoma or renal neoplasms in rodents. 2-Acetylaminofluorene was fed at 0.03 percent of the diet, aminoazotoluene, aminotriazole, carbarsone, DDT, diethylstilbestrol, dimethylnitrosamine, *p*-dimethylaminoazobenzene, thioacetamide, and thiourea at 0.06 percent of the ration, tannic acid at 0.08 percent of the diet, and carbon tetrachloride and urethane at 0.16 percent of the diet for the 1 × level, respectively. The standard laboratory diet used was casein 35, gelatin 15, dextrin 29, vitamins 1, alpha-cellulose 8, minerals 4, cod liver oil 2, corn oil and additives 6, and water 125. Careful examination of over 300 livers of rainbow trout fed up to 1 year on this complete test diet disclosed only one incipient hepatoma and one small hepatoma in any of these samples skip serial sectioned and carefully examined microscopically.

In contrast rainbow trout fed this standard test diet containing a carcinogen at an appropriate level became tumor bearing in from 4 to 9 months of feeding under the experimental conditions used (Halver, Johnson, and Ashley, 1962). As anticipated, at higher levels (16 × and 4 ×) many of these chemical carcinogens showed a profound toxic effect. Most of the groups of fish fed these high levels were terminated because of excessive mortality which appeared between 6 weeks and 6 months of feeding, depending upon the specific chemical involved and the toxic level ingested. Most fish fed lower levels of each of the 13 original compounds did survive, with very specific lesions found in the liver or in other tissues of each group. Results were difficult to treat statistically because of the periodicity of sampling and because all samples which were preserved for histological analysis were not sectioned, stained, and analyzed micro-

scopically. However, a mathematically real incidence of histopathologically confirmed hepatoma did appear in those groups of fish fed intermediate or low levels of 2-acetylaminofluorene, aminoazotoluene, DDT, diethylstilbestrol, dimethylnitrosamine, *p*-dimethylaminoazobenzene, thiourea, tannic acid, carbon tetrachloride, and urethane (Halver, La Roche, and Ashley, 1963). In concurrent studies, fish fed fat carefully extracted and concentrated by the Bloor-La Roche technique (La Roche et al., 1962) from a suspect commercial dry diet showed a significant number of liver nodules after 9 months of feeding the control diet to which the extracted lipid had been added. Histological examination of the nodular livers showed loci of acidophilic or fatty degenerations in either the adenocarcinoma or trabecular type reminiscent of hepato-fibrillar growths in mammalian hepatoma (La Roche et al., 1962).

From these preliminary studies it was concluded (1) that liver cell carcinoma of adenomatous, trabecular, or mixed type would appear in rainbow trout fed an appropriate ration containing a carcinogenic load of a number of different chemical compounds; (2) that visual detection of the small developing nodules could be estimated with reasonable confidence after 6 to 9 months of feeding the carcinogens; (3) that the growth rate of individual or multiple nodules could be estimated under the standard experimental and dietary conditions used; and (4) that rainbow trout were an acceptable experimental animal for oncology. See table 40.

Another vector in the appearance of fish hepatoma may be the relation between oxidized fats, ceroid, and hepatomagenesis (Scolari, 1954; Wood and Yasutake, 1956; Ghittino and Ceretto, 1962). Fishery investigators in Italy described tumors in rainbow trout and more recently in brook trout (*Salvelinus fontinalis*) which had livers containing a typical yellow-red sudano-

TABLE 40.—*Hepatoma I series—Gross tumor index*

Compound	Dose	15 months	20 months	Histology
Urethane	1280 mg%	2/49	5/55	yes
Do	5120 mg%	3/50	4/38	yes
Carbon tetrachloride	320 mg%	3/45	4/44	yes
Do	1280 mg%	3/28	3/34	yes
Carbarzone	480 mg%	1/49	5/50	yes
Do	1920 mg%	2/2	2/39	yes
Thioacetamide	480 mg%	0/15	---	no
Do	1920 mg%	1/15	---	no
Aminotriazole	120 mg%	0/48	---	no
Do	480 mg%	0/57	---	no
2-Acetylaminofluorene	15 mg%	13/58	9/45	yes
Aminoazotoluene	30 mg%	6/54	3/49	yes
Do	120 mg%	10/46	22/43	yes
Dichloro-diphenyl-trichloroethane	7.5 mg%	7/19	4/11	yes
p-dimethylaminoazobenzene	7.5 mg%	5/56	8/55	yes
Do	30 mg%	4/52	6/46	yes
Thiourea	120 mg%	4/40	12/42	yes
Do	480 mg%	3/32	7/38	yes
Dimethylnitrosamine	480 mg%	33/81	38/51	yes
Do	1920 mg%	65/65	---	yes
Diethylstilbestrol	1.875 mg%	3/3	dead	yes
Tannic acid	160 mg%	4/48	6/48	yes
Do	640 mg%	4/54	4/52	yes
Complete test diet	---	0/100	0/300	no
Total lipid	6%	25/100	5/11	yes
Protein	25%	6/48	2/41	yes
Carbohydrate	7%	0/100	6/68	yes

philic compound subsequently identified as ceroid (Ghittino, 1961, 1963; Ghittino and Leon, 1963). This material may appear, dependent upon age and the dietary, in sporadic fashion throughout the parenchymal cells of fish liver. It may occur after continuous ingestion of partially oxidized polyunsaturated fatty acids without adequate intracellular antioxidants such as *alpha* tocopherol, and in the latter cases became so exaggerated as to occupy most of the intracellular spaces and obviously impair normal liver function. While some specific causative agents for the appearance of ceroid are known, (Wood and Yasutake, 1956; Horwitt et al., 1961) the extent and nature of the condition and environmental and dietary treatments which may reverse and repair the livers is currently the subject for intensive investigation. Very recently epicarditis and ceroid deposition in the spleen have been demonstrated in *alpha* tocopherol deficient chinook salmon, (Woodall et al., 1964) and evidently the protective action of *alpha* tocopherol as an intracellular antioxidant has become more firmly established for the fish family Salmonidae.

Acute toxicity was reported for *Aspergillus flavus* (Link) contaminated peanut meal fed to turkeys, ducklings, chickens, cattle, sheep, hogs, and rats (Blount, 1960; Wiley, 1960; Allcroft et al., 1961; Carnaghan and Sargeant, 1961; Lancaster, Jenkins, and Philp, 1961; Loosmore and Harding, 1961; Loosmore and Markson, 1961; Wannop, 1961; Archibald, Smith, and Smith, 1962; Forgacs et al., 1962; Kohler and Swoboda, 1962; Paterson et al., 1962; Richardson et al., 1962; Smith and McKernan, 1962; Allcroft and Lewis, 1963a, 1963b, Aust et al., 1963; Blakely et al., 1963; Blount et al., 1963; Harding et al., 1963; Hartley, Nesbitt, and O'Kelly, 1963; Spensley, 1963.) Forgacs and Carll (1962) and Kraybill and Shimkin (1964) summarized the general mycotoxicosis problem. Long-term chronic feeding studies showed permanent liver damage and implicated hepatomagenesis in rats and guinea pigs (Schoental, 1961; LeBreton, Frayssinet, and Boy, 1962; Salmon and Newberne, 1963). Chemical structure determinations, methods of separation of crude aflatoxin, and chromatographic separation of aflatoxins B and G made possible preparation of milligram quantities of the aflatoxins by Wogan and others of the MIT group for long-term trout hepatomagenesis studies (Wynston and Tilden, 1962; van der Zijden et al., 1962; Asao et al., 1963; Carnaghan, Hartley, and O'Kelly, 1963; Chang et al., 1963; Coomes and Sanders, 1963; Wogan et al., 1963).

In preliminary experiments with fish, crude aflatoxin concentrates were force fed to 50-gm rainbow trout in five daily doses of 0.8, 8.0, and 80 mcgms respectively. By day 7, 8 of the 10 fish dosed at the high level were dead. Histopathological examination confirmed typical massive hemorrhagic necrosis of liver parenchyma with little or no proliferation of bile ducts or cholangioles. A comparison of the results with high load test duckling assays indicated similar but more extensive typical damage of liver parenchyma in the trout. Trout liver slides and duckling liver slides of animals of the same weight on the same load test were carefully compared. Grossly typical massive liver hemorrhagic areas were seen with some other hemorrhagic areas extending throughout the viscera. Other effects observed in the peritoneal cavity were so dramatic that the load test had obviously exceeded an LD_{50} level and did serve as the basis for subsequent and more definitive studies.

In the second experiment crude afla-

toxin was force fed to 50-gm trout at 5 mg/kg body weight, 3 mg/kg body weight, 1 mg/kg body weight in single dose and at 1 mg/kg body weight daily for 5 days, respectively. The crude aflatoxin was dissolved in 95 percent ethanol, was spread over an appropriate quantity of α-cellulose, was dried overnight at room temperature, and was weighed into gelatin capsules. Ten fish were held on each treatment for a 10-day observation period. Results in moribund fish up to the last survivor on day 10 showed all in the 5 mg/kg group had gross multiple hemorrhagic areas throughout the liver and other viscera. Hemorrhagic areas in the liver were interspersed with hard firm yellow tissue and in the group fed 3 mg/kg body weight single dose, all fish had multiple hemorrhagic areas in the liver and viscera. Most fish were moribund by day 10. No normal appearing livers were seen in this group of the five survivors examined. In the group force fed 1 mg/kg body weight single dose, multiple small hemorrhagic areas involving about half the liver were observed. Generally, the hemorrhagic areas were found on the liver extremities. Six of 10 fish survived the 10-day experiment, all survivors had obvious severe damage. Also in fish fed 1 mg/kg body weight per day for 5 days, 6 fish survived the 10-day experimental period. All survivors had multiple hemorrhagic areas in the liver interspersed with hard, firm yellow tissue and multiple hemorrhagic areas in fatty portions of other viscera. All survivors were obviously moribund. In contrast, the controlled diet fed fish had healthy normal appearing viscera with normal liver color and texture.

On the basis of these preliminary experiments the LD_{50} dose rate for 50-gm rainbow trout appeared to fall between 1 and 3 mgs of the crude aflatoxin concentrate per kg body weight. Subsequently a small shipment of crystalline aflatoxin B_1 and crystalline aflatoxin G_1 was obtained from Dr. Wogan of the Massachusetts Institute of Technology. Crystalline aflatoxin was dissolved with 2 mls of chloroform, was mixed thoroughly with 2 gms of *alpha* cellulose flour, and was dried overnight at room temperature to remove chloroform vapors. Small quantities of this mixture were diluted with *alpha* cellulose flour, mixed thoroughly, checked under long-wavelength ultraviolet light, and appropriate quantities were packed in gelatin capsules to treat 10 fish each at 0.1 mg/kg body weight, 0.5 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, for single dose treatments; and 10 fish at 0.1 mg/kg body weight per day for 5 days and 1 mg/kg body weight per day for 5-day treatments, respectively. For comparison purposes, sham treated controls received *alpha* cellulose flour to which the maximum quantity of chloroform had been added and removed by overnight drying before loading and feeding. Results of the high load tests feeding experiment are summarized in table 41. These preliminary studies indicated acute gross toxicity of either crude or crystalline aflatoxins in small rainbow trout and long-term chronic load tests were designed for trout hepatomagenesis studies.

TABLE 41.—*High load aflatoxin tests*

Dose	Day 10 survival	Liver gross damage
0.1 mg B_1 crystalline	10	+
0.1×5	5	+++
0.5	5	+++
1 mg	[1] 9	+++
1 mg×5	0	+++
3 mg	[1] 7	+++
1 mg crude	10	+
1 mg×5	7	++
3 mg	8	+++
5 mg	10	+++

[1] All moribund; moribund fish noted would obviously have expired from extensive internal damage seen on necropsy.

The above preliminary hepatoma induction experiments indicated that eleven chemical compounds could be used to induce an incidence of parenchymal cell carcinoma in rainbow trout, but the dose response was poorly defined and a second series of experiments was initiated to measure the incidence of hepatoma that would appear on effective but intermediate dietary load levels of most of the 11 classical carcinogens. In addition, thioacetamide and aminotriazole were reexamined. Also by this time the total fat extracted from the commercial ration had been successfully separated on large chromatography columns into neutral lipid and phospho lipid components. These were in turn included in the design of the induction experiments, and were incorporated into the test diet at that level found in the suspect commercial pellet. For comparison purposes the protein and carbohydrate fractions were again tested, and were compared with fish fed the original suspect pellet, and the complete test diet basal ration. Surgical inspection on samples of the populations of rainbow trout on test were completed after 9 months of feeding; then duplicate lots were combined and were transferred to 6-foot circular tanks for extended feeding studies. Representative tissues observed were carefully preserved for subsequent histological analysis. Populations on test were cut to 100 fish on each treatment because of limited space and facilities available. After 12 months on test each survivor was inspected and the surgical gross index (SGI) of tumors was established.

This procedure was repeated at 16 months on test, and the SGI of survivors was repeated on necropsy after 20 months on experiment. Results of the change in numbers of tumors seen in the population can be seen in the population tabulated in table 42. One may note the excellent survival of individual fish on treatment in each lot for most of the groups studied. One should also note that samples removed for subsequent histological analysis at the 12-month inspection period reduced the total population by 10 for 16-month inspection. These included at least five tumor bearing livers and at least five suspect livers from each treatment (if available). Each liver anomaly selected was carefully described and preserved for histological analysis at the 12-month inspection period. This would tend to increase the percentage of tumors in the original population over that found at the 16-month and 20-month inspection periods. At necropsy up to 10 classical tumors and up to 10 suspect livers were carefully preserved for subsequent histological analysis. In addition, 10 apparently normal livers were carefully preserved and were reviewed histologically to establish a microscopic incidence of small tumors which might be present in this segment of the population at the 20-month termination period. Consistent throughout this experiment was the absence of tumors in those groups fed the complete test diet basal ration.

Hepatoma III induction diets contained crystalline aflatoxin B_1 at 0.5 ppb, 2.0 ppb, 8.0 ppb; crystalline aflatoxins b and g extracted from *A. flavus* contaminated peanut meal at 10 times these levels; and a crude aflatoxin concentrate recovered by Dr. Wogan from *A. flavus* cultured on shredded wheat at 1.25 ppb, 5 ppb, 20 ppb, 80 ppb, and 320 ppb. All aflatoxin compounds were absorbed on *alpha* cellulose flour and incorporated at the above levels into the complete test standard reference diet. Each treatment was fed to duplicate lots of 100 initial feeding rainbow trout under standard hepatoma induction experiment conditions (15°C). After 6 months of feeding, random samples of

TABLE 42.—*Hepatoma II series—Surgical gross index*

Compound	9 months				12 months				16 months				20 months			
	Number	Normal	Suspect	Tumors	Number	Normal	Suspect	Tumors	Number	Normal	Suspect	Tumors	Number	Normal	Suspect	Tumors
AAF 15 mg%	42	42	--	--	101	85	10	6	85	75	9	1	83	73	5	5
60 mg%	30	24	5	1	99	82	10	7	82	72	8	2	81	72	5	4
AAT 30 mg%	40	39	--	1	99	81	9	9	78	67	4	7	77	55	15	7
120 mg%	42	40	--	2	98	57	33	8	83	43	20	20	77	40	21	16
DDT 1.8 mg%	40	39	--	1	81	77	3	1	50	37	10	3	58	49	6	3
7.5 mg%	26	22	4	--	72	65	4	3	65	54	6	5	33	22	9	2
p-DAAB 7.5 mg%	40	35	4	1	99	83	12	4	63	47	9	7	28	19	2	7
DMN 30 mg%	29	29	--	--	100	50	8	42	81	42	6	33	64	12	3	49
120 mg%	44	41	2	1	101	32	10	59	87	32	8	47	79	8	1	70
Carbarsone 30 mg%	47	45	1	1	99	87	5	7	86	76	7	3	78	64	6	8
120 mg%	40	36	3	1	92	77	10	5	83	73	5	5	69	43	16	10
TAA 30 mg%	48	44	3	1	100	67	19	14	77	45	25	7	64	39	23	2
120 mg%	35	32	3	--	99	46	25	28	76	33	25	18	45	26	14	5
ATA 120 mg%	17	13	--	4	73	45	13	15	47	34	8	5	35	28	2	5
480 mg%	9	2	2	5	48	31	12	5	34	21	9	4	33	25	6	2
CTD	25	23	2	--	96	89	6	1	92	86	6	--	71	59	11	1
Total fat 6%	47	17	19	11	99	34	29	36	46	12	14	20	45	4	13	28
Neutral lipid 5%	38	33	2	3	98	62	19	17	85	58	14	13	80	52	21	7
Phospho lipid 1%	33	33	--	--	100	88	9	3	90	69	18	3	84	66	13	5
Protein 25%	47	43	2	2	97	75	19	3	84	75	6	3	84	70	10	4
Carbohydrate 7%	40	33	7	--	99	68	25	6	85	59	18	8	85	46	30	9
Pellet 100%	52	47	4	1	100	78	11	11	88	64	8	16	82	46	15	21
Oxidized herring oil 6%	38	31	7	--	98	71	12	15	82	55	17	10	63	40	15	8
Stripped herring oil 6%	43	37	5	1	98	83	7	8	88	77	7	4	61	53	3	5

livers from fish from each subgroup were removed for subsequent histological assay. Occasional small incipient nodules were found and on the basis of the best evidence at that time from necropsy inspection of only small numbers of fish, those groups fed crystalline aflatoxin B_1 at 0.5 ppb, crystalline aflatoxin G_1 at 0.5 ppb, aflatoxins B and G at 5 ppb, and crude aflatoxin at 1.25 ppb and 5 ppb in the dietary, were sampled extensively for histology and were deleted from the experiment because of space limitations at the Hagerman Nutrition Laboratory bioassay site. After 1 year on test each fish in each lot was surgically inspected. All tumors or other anomalies observed in the liver or other internal organs were carefully described and each fish was identified with a numbered spaghetti tag before return to the recovery tank.

Suspect or typical examples of grossly visible tumors were deliberately selected and carefully preserved for subsequent histological analysis. Almost all the selected nodule samples were confirmed histopathologically as hepatoma. In addition to the crystalline and crude aflatoxin components included in this experiment, hepatoma II induction experiments had indicated the possibility that the neutral lipid component, separated from the total fat from suspect commercial ration might contain material involved in rainbow trout hepatomagenesis. This neutral lipid component was fractionated on silicic-acid-powered glass columns, using mixtures of hexane and diethyl ether into *alpha, beta, gamma, delta,* and *epsilon* subfractions. These were also incorporated into the design of the experiments at that level found in the suspect commercial ration. For comparison purposes the original total fat, the neutral lipid fraction, the whole pellets, and the DMN positive standard were also fed to individual groups of rainbow trout. These in turn were surgically inspected after 12

TABLE 43.—*Hepatoma III series—Surgical gross index*

Compound	12 months				16 months			
	Number	Normal	Suspect	Tumors	Number	Normal	Suspect	Tumors
B_1 2 ppb	74	69	3	2	64	53	6	5
B_1 8 ppb	75	68	5	2	24	20	3	1
G_1 2 ppb	72	48	13	11	55	39	11	5
G_2 8 ppb	74	52	14	8	58	39	12	7
b_1 20 ppb	78	13	15	50	62	7	9	46
b_1 80 ppb	76	10	17	49	64	5	13	46
g_1 20 ppb	88	80	5	3	74	72	2	--------
g_1 80 ppb	68	62	6	--------	55	42	13	--------
Crude 20 ppb	115	78	19	18	109	73	12	24
80 ppb	106	19	11	76	105	23	4	78
320 ppb	113	66	8	39	119	59	9	51
C.T.D.	110	108	2	--------	50	49	1	--------
Total fat 6%	107	63	21	23	51	17	13	21
N L	69	56	4	9	48	30	17	1
Pellets	87	62	10	15	54	17	8	29
Alpha	77	62	10	5	47	31	10	6
Beta	86	79	5	2	39	32	7	--------
Delta	83	75	7	1	46	40	6	--------
Gamma	87	76	8	3	49	42	6	1
Epsilon	85	83	1	1	49	47	1	1
DMN 30 mg%	113	94	9	10	89	74	4	11

months, and after 16 months on test. The surgical gross index of tumors observed at these two inspection periods can be seen in table 43.

At the highest dose levels of crude aflatoxin (320 ppb), microscopic analysis disclosed consistent toxic liver damage which may be reflected in the apparent lack of tumor response when this crude aflatoxin was fed at 320 ppb. At 80 ppb load level, however, over 50 percent of tumor bearing trout were found on 1-year inspection. At lower and more appropriate hepatogenic dietary load levels, response did appear over the range of treatments used. Unfortunately subsequent histological analysis of the 6-month samples of trout fed only 0.5 ppb crystalline aflatoxin B_1 disclosed a real incidence of micronodules of classical trabecular hepatoma, but these groups had been discarded in favor of space considerations.

Typical trabecular hepatoma, with occasional metastasis to other organs such as the kidney, gills, ovaries, and oftentimes invasion of surrounding tissue including the musculature, was frequently encountered during more extended feeding trials. The hepatoma tumors encountered in those groups fed either crude or crystalline aflatoxins appeared similar to those observed in fish fed the original suspect ration, the total fat extracted from the ration, and from the *alpha* fraction separated from the neutral lipid from the fat from the feed.

Samples of the total lipid extracted from suspect commercial ration, and which was incorporated into the diet at 6 percent of dry diet ingredients, was positive for tumorigenesis in hepatoma I, II, and III feeding experiments.

Samples of this total lipid were shipped to MIT and intubated into day-old ducklings. Subsequent histological analysis of the livers after the 7-day test disclosed some slight liver damage but damage *not* typical for the aflatoxins. Analysis of small samples of total lipid and *alpha* fraction for aflatoxin by chemical methods failed to disclose minimum detectable amounts, although some fluorescent material which would migrate near to that of aflatoxin B_1 was present in the samples. From organic chemistry considerations, and from separation of total lipid into neutral lipid and then into *alpha*, *beta*, *gamma*, *delta*, and *epsilon* fractions, with relatively nonpolar solvent systems, it appeared that, if aflatoxins were the primary vector for hepatomagenesis in the *alpha* subfraction from the neutral lipid fraction, then the aflatoxin must have been altered in processing or during storage to become less polar in nature in order to travel with the relatively nonpolar neutral lipid subfractions.

A sample of crude aflatoxin obtained from laboratories at MIT was dissolved in chloroform: diethyl ether and thoroughly mixed with the *alpha* fraction from the neutral lipid fraction from the total lipid from the feed. Chloroform and ether were removed under reduced pressure in a rotary evaporator. The *alpha* fraction containing the crude aflatoxin was dissolved with dry ethyl-ether and added to the silicic acid: powdered glass column commonly used for fractionation of the neutral lipid fraction into *alpha*, *beta*, *gamma*, *delta*, and *epsilon* subfractions. At this point twice the normal amount of dry ethyl-ether necessary to elute the neutral lipid subfraction was used, and then was followed with absolute methanol in the quantity used for removing the phospho lipid fraction from the fat from the feed. The progress of the solute could be followed under ultra-violet light and discrete characteristic fluorescent bands were traced during

the elution process. The blue and green fluorescent compounds in the crude aflatoxin stayed on the column during the ether elution although some widening of the bands did occur when these large volumes of ether were passed through the column. None appeared to migrate down the 2 meter column into the ether soluble subfraction, and upon evaporation no material with proper activation and emission energies was observed. In contrast, all the blue and green compounds were rapidly eluted when methanol was added to the column and concentration disclosed a substantial portion of the aflatoxin in this eluant. Therefore, it would be probable that any unaltered aflatoxin present in the suspect commercial ration or in the total lipid fraction from this ration would have been retained on the column, and then eluted in the phospho lipid phase, unless these aflatoxins were conjugated on very closely associated with some nonpolar constituents. Subsequent analyses of the original suspect ration, of the total lipid fraction of the ration, and of the protein residue left after extraction with Bloor-La Roche solvents have been analyzed for aflatoxin content.

The nature of the suspect ration, containing many dietary components including cottonseed meal, distillers solubles, and large amounts of fish meal, bone meal, blood meal, salmon oil, polymerized fat, and greases, presented difficult problems to the analyst. Many masking migrating fluorescent compounds appeared on thin layer chromatography of solvent extracts from this feed. The Southern Regional Laboratory has reported, however, significant concentrations of aflatoxins in the total lipid and protein residues indicating only partial extraction of the aflatoxins by the Bloor-La Roche technique.

Current major efforts in Hepatoma Induction IV experiments were designed (1) to lower the insult in the dietary of both crystalline aflatoxin B_1 and crystalline aflatoxin G_1 (2) to retest phospholipid components in the feed as a possible site for pure aflatoxin residues and (3) to study fats extracted from cottonseed meal processed by three standard industrial methods and currently used in commercial feed formulations. In addition, commercial fat polymers were tested both with and without (1) 2-acetylaminofluorene and (2) dimethylnitrosamine.

Preliminary experiments with Dr. Miller at McArdle Research Foundation in Wisconsin on fish cannulated with a ureter and force-fed loaded doses of 2-acetylaminofluorene showed 7-hydroxy and 5-hydroxy AAF intermediates in the urine, but a very low potential for converting 2-acetylaminofluorene into the N-hydroxy derivative. Therefore, both 2-acetylaminofluorene at 15 mg percent and N-hydroxy acetylaminofluorene at 3 mg percent were included in the dietary treatments. The same general experimental technique was followed with duplicate lots of 100 fish fed the various dietary treatments in constant temperature water (15° C.) for 6 months. At that time representative random samples of liver tissue were carefully prepared for subsequent histological analysis, all populations were reduced to 100 fish each, and the combined lot on each treatment transferred to 6-foot circular tanks for the extended feeding study. After 1 year on test all animals were surgically inspected. Liver and other internal anomalies were carefully described. Each fish was identified with a numbered spaghetti tag before return to the recovery tank, and a SGI of tumors in the individual population was assembled. See table 44.

A high incidence of classical trabecu-

TABLE 44.—*Hepatoma IV series—Surgical gross index at 1 year*

Compound	Number	Normal	Suspect	Tumors
B_1 0.5 ppb	116	47	32	37
B_1 2 ppb	115	25	45	45
B_1 8 ppb	121	30	39	52
G_1 0.5 ppb	114	87	22	5
G_1 2 ppb	127	81	33	13
G_1 8 ppb	57	24	22	11
Crude 20 ppb	117	38	35	44
C.T.D.	77	68	8	1
P.L.	117	105	11	1
C.S.M.—1	115	103	10	2
C.S.M.—2	116	92	13	11
C.S.M.—3	111	103	8	
C.S.M.—4	104	92	10	2
C.S.M.—5	114	99	13	2
C.F.P. .0675	109	96	10	3
C.F.P. .25	112	107	4	1
C.F.P. 1%	111	91	13	7
C.F.P.+AAF	107	104	2	1
C.F.P.+DMN	104	93	7	4
K.P.F. 0.25%	81	75	8	3
DMN 15 mg%	114	103	8	3
AAF 15 mg%	113	99	14	
N-OH-AAF 3 mg%	110	106	4	

lar hepatoma was observed in those groups fed crystalline aflatoxin B_1 at even 0.5 ppb (0.5 mcgm/kg diet). Fish after 1 year on test diet weighed about 150 gm each. Conversion of food was conservatively estimated at 2 to 1 which would extend to a total dietary intake of approximately 0.15 mcgm of crystalline material. This small intake was apparently sufficient for tumor formation in about one-third of the population in one year.

ACKNOWLEDGMENT

Obviously this material on aflatoxicosis in rainbow trout was the result of the mobilized activities of the entire Western Fish Nutrition Laboratory and all cooperating research institutions engaged in rainbow trout hepatoma research. These included agencies of federal and state governments, a number of universities, and other research institutions both here and abroad. Assessment of importance of specific contributions from each researcher involved is indeterminable and all are sincerely acknowledged.

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FIGURE 35.—Normal liver in rainbow trout fed 1 year on control diet.

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Discussion

DR. HALVER: The last time we were here we reported our initial study which showed that fish do get tumors; that fish fed 11 different classes of carcinogens over a 256-fold range would respond at an appropriate level, with liver tumors being formed; that these tumors were hepatoma at one stage or another; that a commercial diet which we obtained was hot, in that when fed, tumors would appear; and that fat from this diet when fed in our complete test diet also was a vector in hepatomagenesis.

Since that time the program has changed and I will try to summarize briefly the current status of our research and what we have found since 1962.

Figure 35 is a normal liver in rainbow trout.

Table 45 shows the diet we used as a basal ration for our carcinogen induction diet studies. This diet was used for studies of additions of known carcinogens, and is a basal ration which we have fed for 8 years to three generations of rainbow trout with the resultant type of livers in figure 35.

Trout do have tumors that are either the single type seen in figure 36, the multiple type shown in figure 37, or the massive necrotic type seen in figure 38.

Fat extracted from a suspect ration fed at 6 percent of the diet, in our test diet, resulted in tumors like those seen in figure 39.

At the time of the last hepatoma research conference we gave you tentative reports and raw data which indicated that 11 chemical carcinogens would work in rainbow trout. These have all been confirmed. We received a yes

TABLE 45.—*Reference complete test diet for fish nutrition studies*
Western Fish Nutrition Laboratory
Cook, Washington

Complete Test Diet

Vit.-free casein	38	parts
Gelatin	12	"
Corn oil	9	"
White dextrin	28	"
a-cellulose mixture	9	"
a-cell 8		
Vitamins 1		
	9	
Mineral mix	4	"
Water	200	"
Total diet as fed	300	"

Vitamin Mix

Thiamin HCl	5 mg
Riboflavin	20
Pyridoxine HCl	5
Choline chloride	500
Nicotinic acid	75
Ca-pantothenate	50
Inositol	200
Biotin	0.5
Folic acid	1.5
Ascorbic acid	100
Vitamin B_{12}	10 mcgm
Menadione (K)	4 mg
Alpha-tocopherol acetate (E)	40 mg
Beta carotene (A)	2 mg
Activated 7-dehydro-cholesterol (D)	5 mcgm

Added in corn oil for fat soluble vitamin test diet

per 100 gms solids

Mineral Mix

USP XII No. 2	plus
$AlCl_3$	15 mg
$ZnSO_4$	300
$CuCl$	10
$MnSO_4$	80
KI	15
$CoCl_2$	100/100 gms of salt mixture

NOTE: Delete 2 parts alpha-cellulose and add 2 parts CMC for preliminary feeding.

Delete 2 parts corn oil, Beta Carotene and Activated 7-dehydrocholesterol and add 2 parts cod liver oil for complete vitamin test diet.

Dissolve alpha-tocopherol in oil mix.

Add Vitamin B_{12} in water during final mixing.

DIET PREPARATION:

Dissolve gelatin in cold water. Heat with stirring on water bath to 80°C. Remove from heat. Add with stirring—dextrin, casein, minerals, oils and vitamins as temperature decreases. Mix well to 40°C. Pour into containers; move to refrigerator to harden; remove from trays and store in sealed containers in refrigerator until used. Consistency of diet adjusted by amount of water in final mix and length and strength of beating.

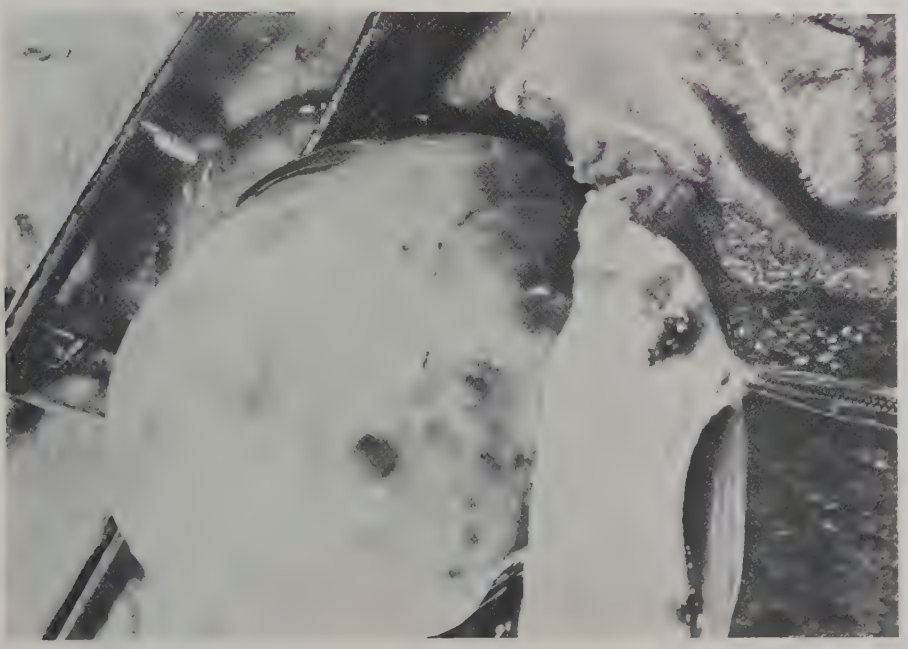

FIGURE 36.—Massive single liver tumor with extensive necrosis and degenerations in center.

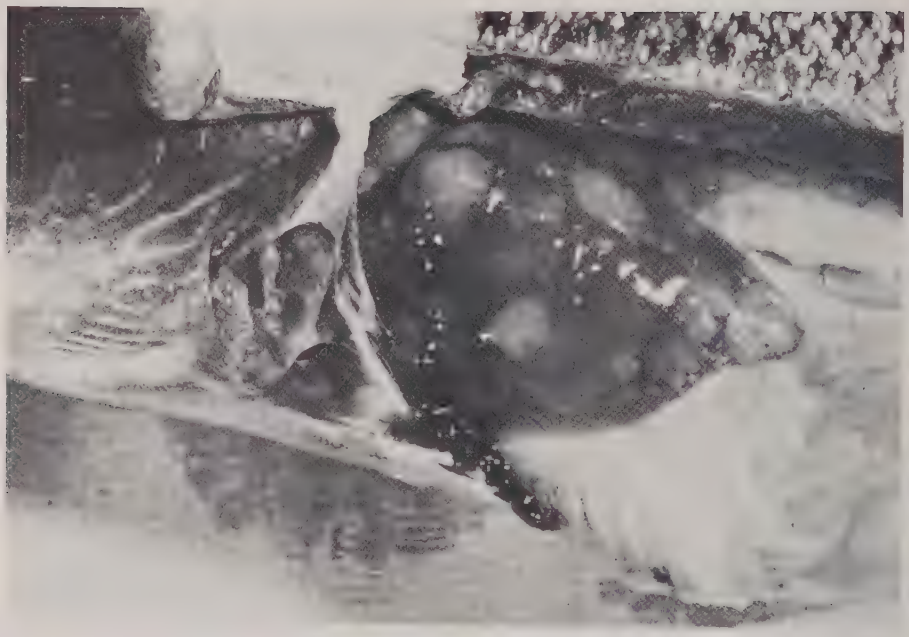

FIGURE 37.—Multiple classical liver tumors in rainbow trout at 1 year on test.

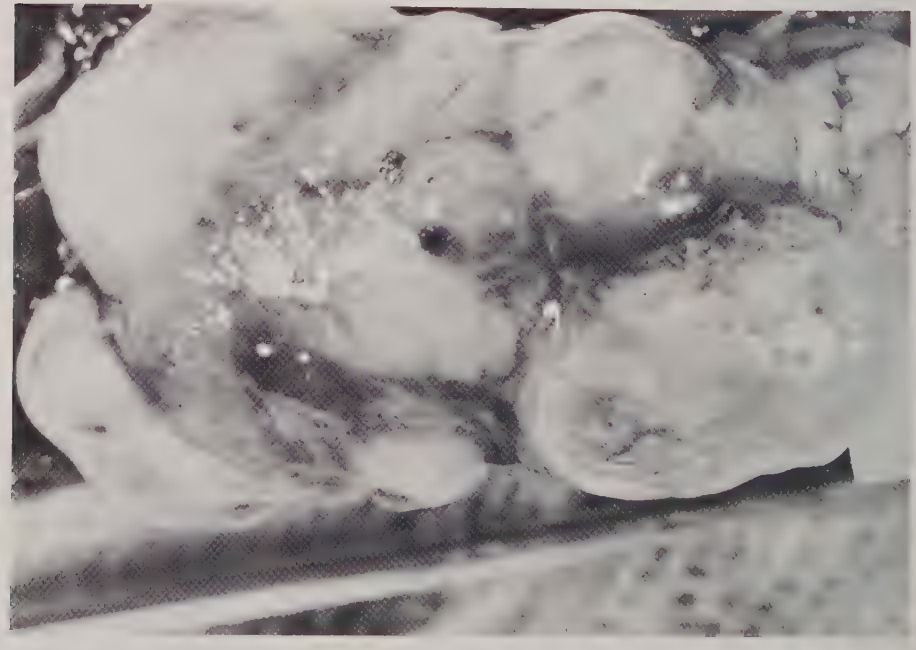

FIGURE 38.—Multiple massive liver tumors with extensive proliferation and extension throughout the viscera.

FIGURE 39.—Two well defined classical liver tumors in trout fed extracted fat at 6 percent of dry diet for 1 year.

answer from 11 of them, and for aminotriazole and thioacetamide we received a no answer shown in table 40.

Figure 40 shows the very gentle Bloor-La Roche technique we used in separating out the lipid from the suspect commercial ration. Final concentration of the lipid material was done in the modified circulating evaporator shown in figure 41.

At the time of these experiments, we did not know about aflatoxin. It was only a word muttered behind closed doors in England and in the ivy covered halls of M.I.T. Therefore we were interested in removing the fat from the ration in the most gentle manner possible. Hence, we used the Bloor-La Roche technique rather than the more drastic Folch technique more commonly used in our laboratory for measuring fat in tissues. We were also conservative regarding chlorinated hydrocarbon solvents, because they were also suspect at this time.

About this time, however, through the urging of Dr. Kraybill and Dr. Shimkin, we did contact Drs. Scrimshaw and Wogan and they were nice enough to give us some of the early aflatoxin concentrates. We tested them in massive doses in rainbow trout to see what would happen. Trout sometimes act like ducklings. They both have nucleated blood cells and they both swim in water. Do they react the same way? They did; in fact the trout reacted better.

Figure 42 shows the result of a single dose of one milligram per kilogram body weight of crude aflatoxin in a 50-

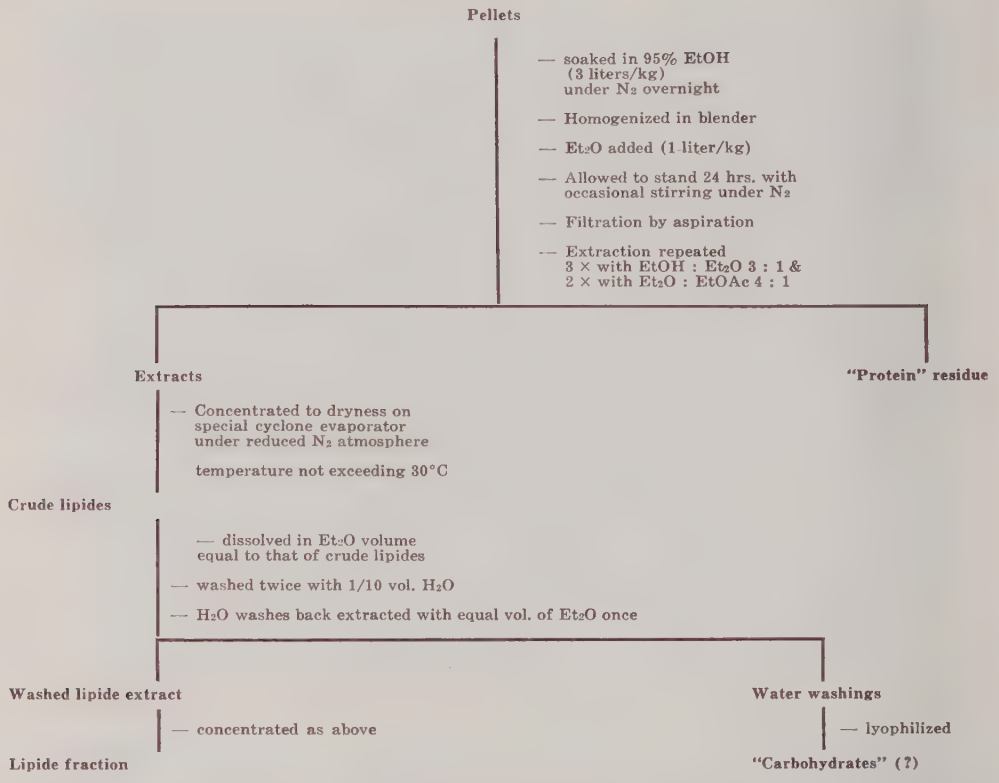
Pellets
— soaked in 95% EtOH (3 liters/kg) under N2 overnight
— Homogenized in blender
— Et2O added (1 liter/kg)
— Allowed to stand 24 hrs. with occasional stirring under N2
— Filtration by aspiration
— Extraction repeated 3 × with EtOH : Et2O 3 : 1 & 2 × with Et2O : EtOAc 4 : 1
Extracts
"Protein" residue
— Concentrated to dryness on special cyclone evaporator under reduced N2 atmosphere
temperature not exceeding 30°C
Crude lipides
— dissolved in Et2O volume equal to that of crude lipides
— washed twice with 1/10 vol. H2O
— H2O washes back extracted with equal vol. of Et2O once
Washed lipide extract
Water washings
— concentrated as above
— lyophilized
Lipide fraction
"Carbohydrates" (?)

FIGURE 40.—Bloor-La Roche technique used to fractionate suspect commercial ration into "fat," "carbohydrate," and "protein" components.

gram trout. Extensive necrosis and hemorrhagic areas are obvious.

Another view of the same in figure 43 shows a little different pattern which is encountered. This particular crude aflatoxin was dissolved in ethanol adsorbed on *alpha* cellulose flour and was incorporated in gelatin capsule and was force fed to an anesthetized 50-gram rainbow trout once at 1 mg/kg body weight. This liver damage seen above in figures 42 and 43 occurred by day 8 after challenge.

The dose was one milligram of the crude concentrate, which contains approximately 30 percent of aflatoxin B_1 plus some B_2, G, and G_2 constituents.

We fed this material for nine months at the rate of 20, 40, and 80 parts per billion of the crude concentrate in the diet and tumors appeared. These were subsequently described as classical rainbow trout hepatoma.

Figure 44 shows two small ones after one year on test. Sometimes one large one, sometimes multiple small ones occur; the same type of multiple distribution that was ordinarily encountered with an effective dose of a chemical carcinogen. Then we obtained some crystalline aflatoxin B_1 and G_1.

We fed one-tenth of a milligram of crystalline aflatoxin B_1 per kilogram body weight per day for five days and wiped out the liver. It was completely necrotic with extensive hemorrhagic

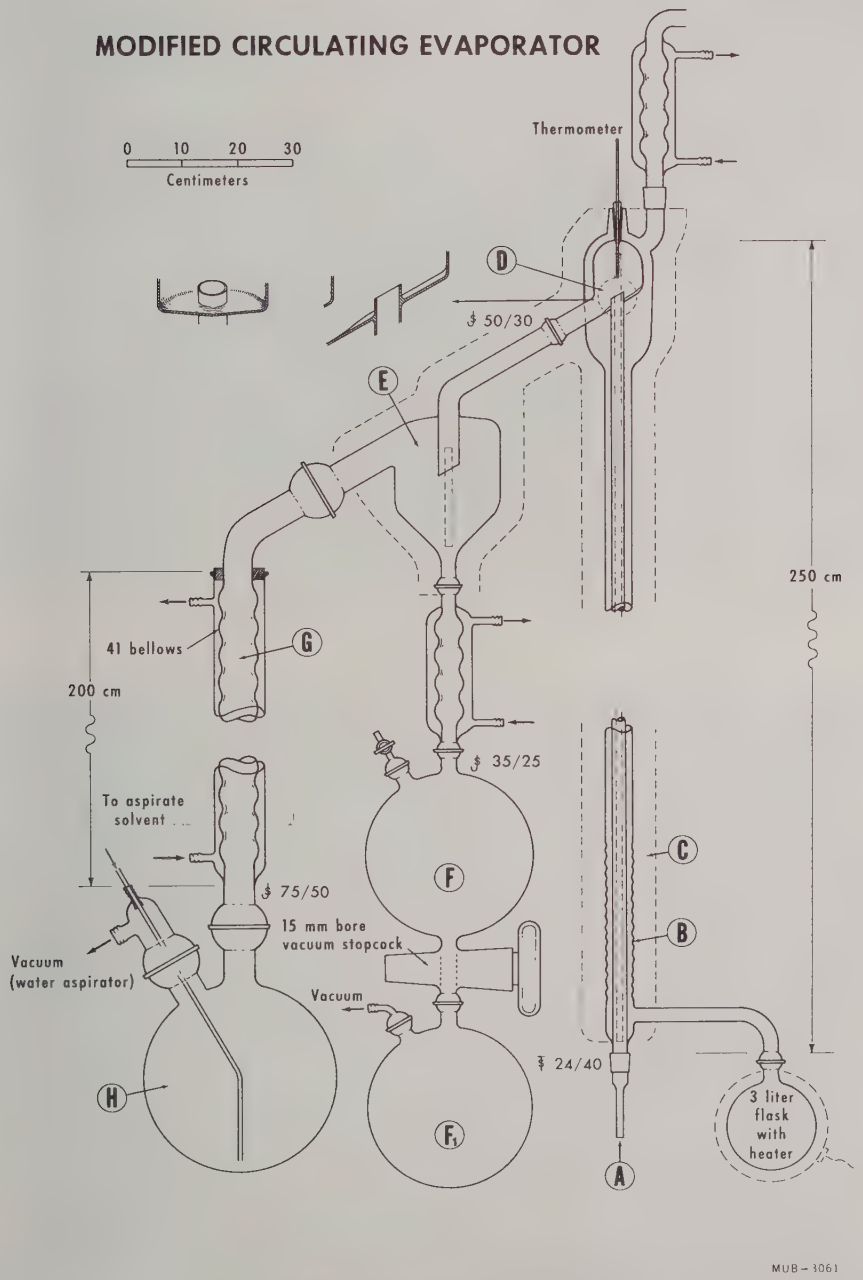

FIGURE 41.—Modified circulating evaporator designed by LaRoche to remove solvents and concentrate lipid fractions for large scale extractions.

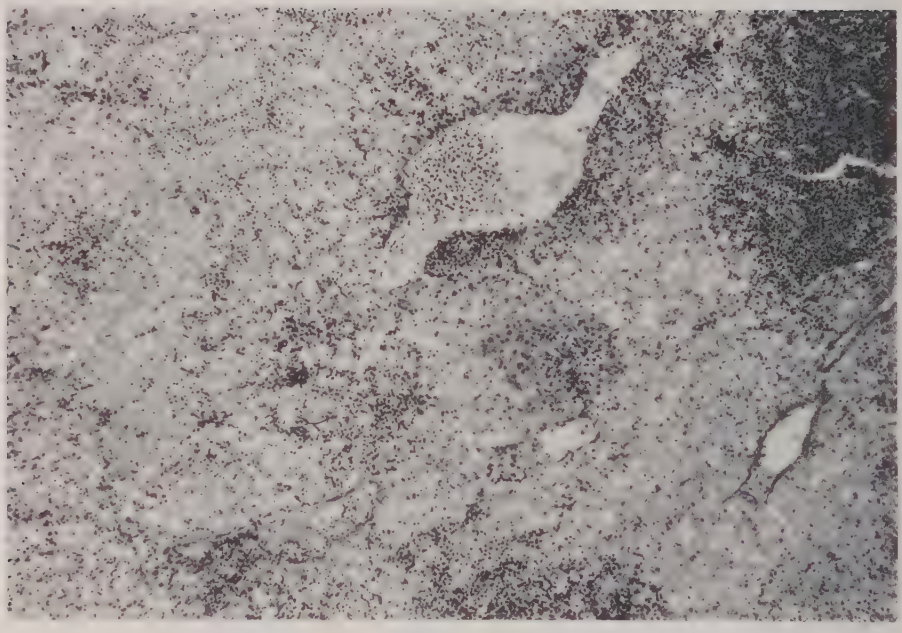

FIGURE 42.—Section of liver after dose of 1 mg/kg body wt. of crude aflatoxin Day 5—moribund 50 gm fish.

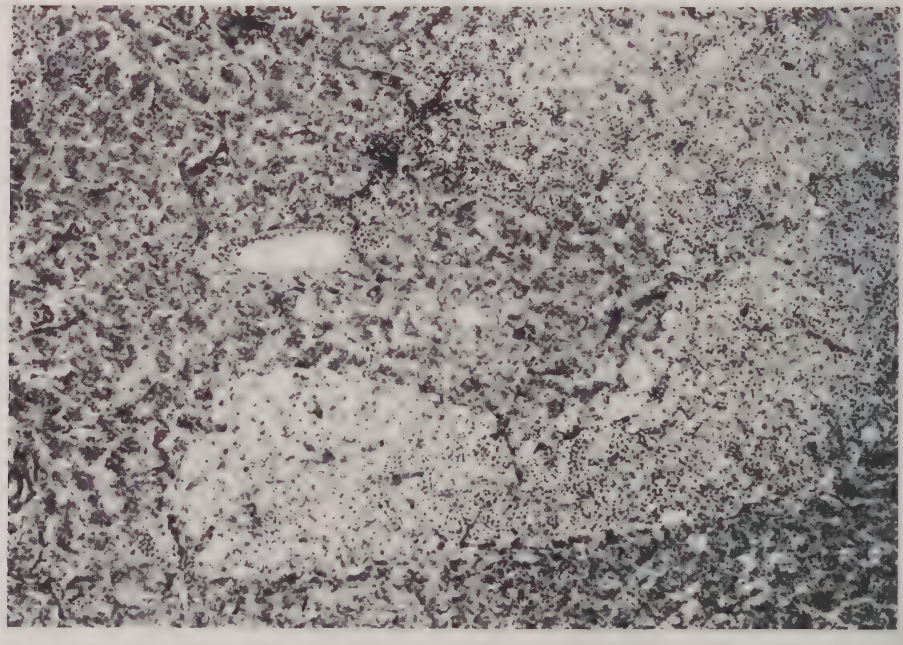

FIGURE 43.—Section of liver from 50 gm trout after dose of 1 mg/kg body weight. Crude aflatoxin. Day 8.

FIGURE 44.—Two classical yellow protruding well vascularized liver tumors in rainbow trout fed crude aflatoxin at 2 ppb in the diet for 1 year.

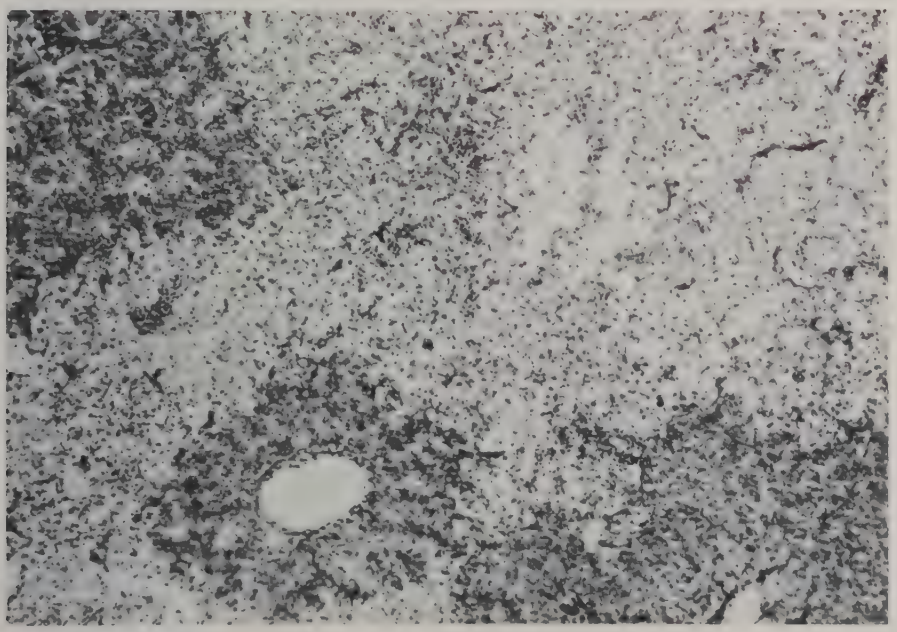

FIGURE 45.—Section of liver from trout fed 0.1 mg/kg body wt/day for 5 days. Aflatoxin B_1. Day 8.

areas throughout the caeca, cecal fat, and other viscera (fig. 45).

Figure 46 shows results of 0.5 milligram per kilogram body weight, single dose on day ten. This fish did last that long. The tentative LD–50 for crystalline aflatoxin B_1 was someplace between four-tenths and six-tenths of a milligram per kilogram body weight, single dose, or administered in five equal doses daily on a 10-day test.

Perhaps the 10-day test is too short, for trout, being poikilothermic, have metabolism slowed down at 15 degree centigrade standard temperatures at which all these experiments were conducted. These fish were all on our complete test diet basal ration for this time period, so we have a standard growth curve.

Perhaps a 3-week test would be more indicative and more comparable to the duckling assay. In the period of time (10 days) the increase in weight of these trout was very small. In the same period of time the duckling will triple in weight.

For comparison, figure 47 shows what a normal liver looks like in the sham treated controls.

The next obvious thing to do after we had this frank and acute toxicity from aflatoxin B_1 in pure form, now that the Wogan factory was in high gear and more material was available, was to start feeding this material. The previous bioassays with the crude material assays showed 1 to 8 parts per billion as a minimum load level. Thus we fed again over an exponential range of 0.5, 2, and 8 parts per billion respectively, to parallel groups of 100 fish each, initial feeding fry, and after 1 year found tumors in the population.

At five-tenths of a part per billion aflatoxin B_1 in the diet, tyical liver tumors developed, subsequently con-

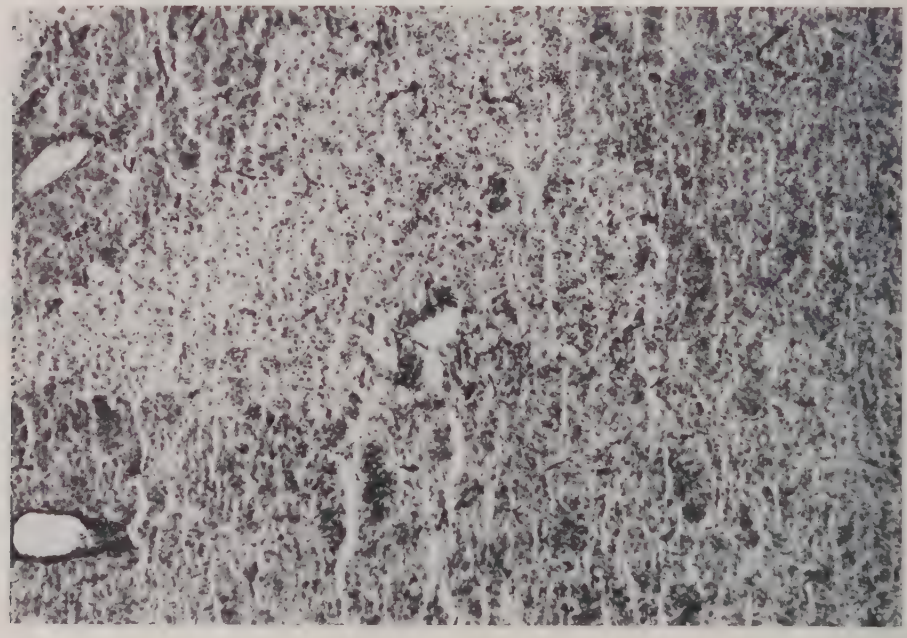

FIGURE 46.—Section of liver from 50 gm trout fed 0.5 mg/kg body weight. Aflatoxin B_1 in single dose. Day 10.

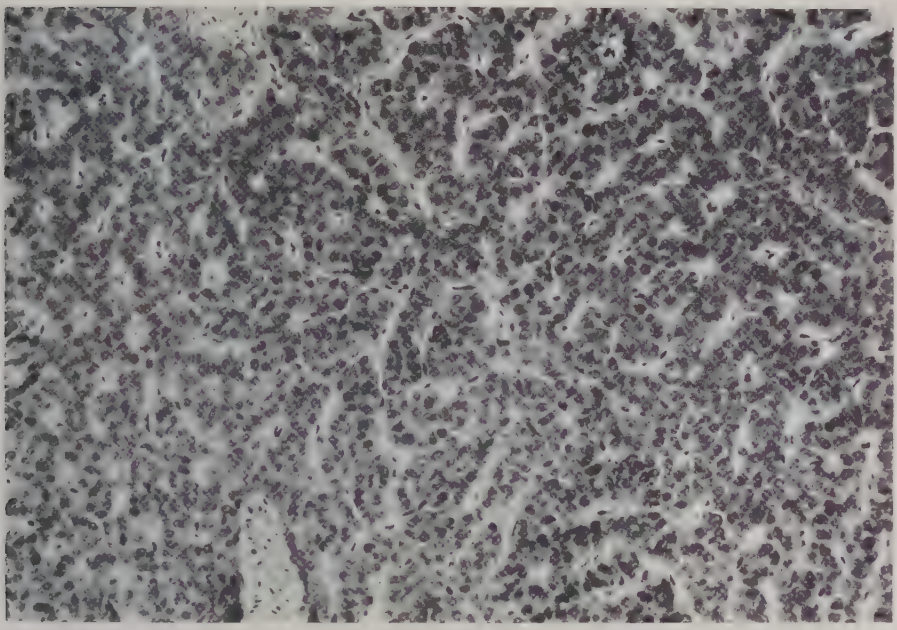

FIGURE 47.—Section of liver from 50 gm rainbow trout force fed alpha-cellulose flour at same level used to contain adsorbed aflatoxin dose treatments.

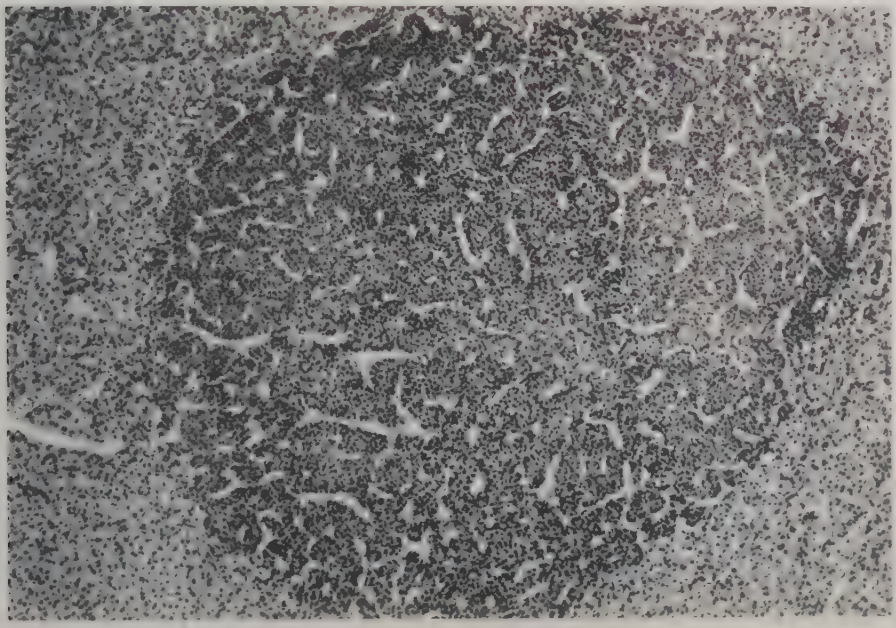

FIGURE 48.—Low power of H. & E. stained liver section from rainbow trout fed aflatoxin B_1 at 0.5 ppb in the diet for 1 year.

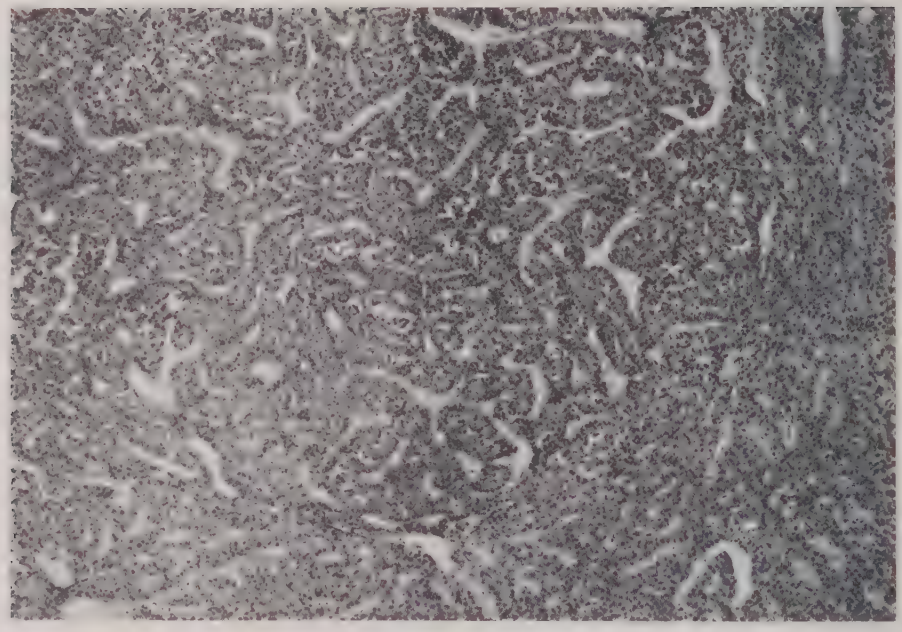

FIGURE 49.—Low power of H. & E. stained liver section from trout fed aflatoxin G_1 at 0.5 ppb in the diet for 1 year.

firmed histologically to be basophilic hepatoma (fig. 48).

Also we fed five-tenths of a part per billion aflatoxin G_1. Small classical type, well-vascularized, yellow, meaty liver tumors developed (fig. 49) but at lower incidences than in those groups fed aflatoxin B_1 at the same dose level.

Suffice it to say that we were able to obtain a very positive and real response with down to one half of one part per billion of crystalline aflatoxin B_1 in the dietary. These fish weighed about 150 grams each. If we very conservatively estimate the food conversion at two to one, that means each fish would have ingested 300 grams of dry diet. At that load level, or even at one part per billion load level there would be about two-tenths of a microgram of material that would have been ingested in this period of time by each individual trout.

This is an area of almost particulate hepatoma induction where mixing becomes a problem, and new and better techniques for homogeneous distribution of these ingredients at these ultra micro quantities will be necessary before we can really measure the minimum effective dose in rainbow trout.

Dr. Wogan just informed me a few moments ago that in our hot diet from which we received a positive response when the diet was fed, when the fat from the diet was fed, or when the neutral lipid fraction from this fat was fed, that one of the samples of this original diet has recently been assayed by his laboratory, and in this particular sample which he received, there were 200 parts per billion of aflatoxin B_1.

I think that's about all I need to say. It looks like one of the causes for trout hepatoma is aflatoxin.

HISTOPATHOLOGY OF RAINBOW TROUT AFLATOXICOSIS

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Rainbow trout of 50 grams average weight, force fed high loads of crude aflatoxin or of crystalline B_1 aflatoxin, had toxicity responses both grossly and histologically comparable to those reported by Newberne et al. [*1*] for 50 gm ducklings fed approximately the same total doses of these aflatoxins. Basic histological lesions in trout differed from those reported for ducklings. While liver parenchyma necrosis and hyperemia (or hemorrhage) were common to both species, bile duct and/or cholangiolar hyperplasia was much more evident in ducklings than in trout. The severity of hepatocellular necrosis and hemorrhage in the two species was similar at the LD_{50} dose, but at higher doses trout appeared to develop more massive necrosis and hemorrhage than was seen in ducklings. It may be conservatively stated, however, that 50 gm Pekin white ducklings and 50 gm rainbow trout are similar in their sensitivity to aflatoxins. Although the liver was most severely damaged in aflatoxic trout, other organs also suffered. These were skin, gills, gastrointestinal tract, pancreas, caeca, visceral fat, spleen, and both hemapoietic and excretory kidneys.

The histopathology of trout aflatoxicosis will now be described, organ by organ.

Liver: The response to LD_{50} and higher doses of aflatoxins, either crude or crystalline B_1, varied from one in which the liver was largely prenecrotic, i.e. diffusely hyperemic with one or more foci of swollen, hypertrophic or pleomorphic parenchyma cells with heavily vacuolated cytoplasm at one extreme, to livers with massive parenchymal necrosis and hemorrhage at the other. Occasional acidophilic (with H & E) cytoplasmic inclusions appeared in vacuolated parenchyma cells but none were found PAS positive in six toxic livers.

Nuclei often contained several small nucleoli or apparently none at all in which case chromatin margination was usually evident. Mitotic figures were rare in parenchymal cells whether cells were normal or degenerative. The least affected parenchyma cells were generally found in the subcapsular region and these as well as others found still intact were Feulgen negative. Parenchymal cells were sometimes grouped as acini around a central lumen, as reported by Newberne et al. [*2*] for ducklings. Frank hemorrhage was seen only in or adjacent to more massive necrotic areas; all others were more or less surrounded by hyperemia where blood was confined to dilated sinusoids and portal veins. Pycnotic nuclei were often abundant adjacent to necrotic foci. Necrotic lesions varied in severity with total dose of aflatoxin fed. The highest hepatoma induction doses of 320 ppb crude aflatoxin fed daily resulted in mildly toxic livers with few

TABLE 46.—*Histopathology of rainbow trout force-fed high loads of aflatoxins*
[Severity of each pathological condition graded on a scale of one plus to three pluses]

Diet load mg/kg body wt.	Vacuolation	Bile duct proliferation	Necrotic foci	Massive necrosis	Hyperemia	Hemhorrage	Pycnotic nuclei	Chromatin margination	Pleomorphism
5 crude	++	−	+	+++	+++	+++	++	++	−
3 crude	++	+	++	+	++	++	++	++	++
1 crude	+++	+	+	−	+	+	++	+	+
3 B_1	++	±	++	+	++	+++	+	++	+
1 B_1	++	+	++	−	++	++	+	+	++
0.5 B_1	++	++	+	−	++	+	+	++	+
0.1 B_1	+++	++	+	−	−	+	+	++	+

or no necrotic cells. Bile duct hyperplasia and cholangiolar proliferation in high load aflatoxin fed trout was variable. The highest load fish rarely had either, but medium load fish had slight amounts while low (LD_{50}) load fish generally had appreciable amounts, the proliferation being often diffuse and poorly differentiated, but in certain cases definitive, proliferating bile ductules were common. Such proliferations in ducklings appeared more often and were more extensive according to Newberne et al. [*1*]. It should be emphasized that necrosis, hyperemia, and hemorrhage appeared to be more severe and extensive in trout than in ducklings. In table 46 the histopathology of aflatoxic trout livers is summarized.

Skin: The dermis was edematous and scale pockets were much dilated in cases with severe toxicosis.

Gills: Hyperemia occurred in variable amounts together with moderate proliferations of lamellar epithelium and moderate clubbing of lamellae.

Swim bladder: The walls were somewhat hyperemic and bloody fluid occurred in the lumen. Adjacent fatty tissues were also hyperemic.

Kidney: Hemapoietic kidney had more or less focal hyperemia seen mainly as engorged and dilated sinusoids. Mild hyperplasia of adrenal cortical tissue was present. This tissue is normally localized around large renal sinuses in teleost fishes. The excretory kidney had increased cellularity of glomeruli with occasional epithelial crescents. A few shrunken glomeruli surrounded by widened Bowman's spaces were also present. Renal tubular lesions akin to those of acute nephrosis occurred but epithelial cell necrosis predominated in collecting or excretory tubules rather than in proximal convoluted tubules as reported for ducklings. Cellular debris was common in degenerating collecting tubules whose epithelial cells had prominent secretory blebs.

Spleen: Hyperemia was severe and reticular proliferations composed of more or less spindled reticulum cells were common.

Pancreas: Acinar cells were more or less hypertrophic and at times markedly desquammated. Adjacent visceral fat had focal hyperemia.

Caeca: Occasionally a diverticulum was found in which necrotic epithelial cells predominated and hyperemia appeared in submucosa and muscularis.

The histopathology of rainbow trout and of Pekin white ducklings exposed to LD_{50} total doses of crude and of crystalline B_1 aflatoxin was similar at least for liver and kidney. Primary lesions in liver, the most severely affected organ, consisted of parenchymal cell necrosis accompanied by hyperemia or hemorrhage. Secondarily in trout, but also primary in ducklings were bile duct and/or cholangiolar hyperplasia. The extent and severity of necrosis in trout exposed to higher than LD_{50} doses

often reached massive proportions, in which cases bile duct proliferations were almost invariably absent. Additional lesions in trout consisted of dermal edema, hyperemia of gills, swim bladder, spleen, hemapoietic and excretory kidney, visceral fat and sometimes of gastrointestinal and caecal walls together with variable necrosis of the epithelium of the gastrointestinal mucosa. Hyperplasia of adrenal cortical tissue was also observed. Pancreatic acinar cells were sometimes hypertrophic and more or less desquammated.

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1964. Histopathologic lesions in ducklings caused by *Aspergillus flavus* cultures, culture extracts and crystalline aflatoxins. *Toxicol. Appl. Pharmacol. 6*:542–556.

NEWBERNE, PAUL M., WILLIAM W. CARLTON, and GERALD N. WOGAN.
1964. Hepatomas in rats and hepatorenal injury in ducklings fed peanut meal or *Aspergillus flavus* extract, Pathologia Veterinaria, *1*:105–132.

Discussion

DR. ASHLEY: Dr. Halver explained something about the use of aflatoxin in our experiments with rainbow trout. As he mentioned we have fed it continuously to replicate 100 fish lots at fairly low levels and in fourfold increments beginning with 1.25 ppb and increasing through 5, 20, 80, and 320 ppb. All these levels were fed with the idea of hepatoma induction in mind.

High load tests involved force-feeding the crude aflatoxin at 1, 3, and 5 milligrams per kilogram body weight, and the crystalline B_1 aflatoxin at 0.1, 0.5, 1.0, and 3.0 mg/kg body weight.

The histopathology of aflatoxicosis, then, can more or less span the entire range of all these levels of aflatoxins. Our figures will illustrate rainbow trout aflatoxicosis beginning with hepatomagenesis and ending with the acute toxic responses observed in various organs.

A relatively normal liver (fig. 50) shows muralia averaging two cells in width which alternate with the sinusoids. A larger vessel (vein) filled with blood is also shown.

Figure 51 shows areas of increased nuclearity and areas of pale, acidophilic, more or less swollen cells, some of which contain bizarre basophilic nuclei, in a preneoplastic portion of a liver which had a tumor nodule at another site. The diet fed was crude aflatoxin at 320 ppb for 9 months.

Two early primary liver cell neoplasms of the classical trabecular type shown in figure 52 are surrounded by heavily vacuolated parenchymal cells typical of many preneoplastic trout livers. Crude aflatoxin was fed at 80 ppb for 6 months. This level also showed a slightly small preneoplastic or hyperplastic basophilic nodule near the smaller hepatoma here but unfortunately it could not be included in the field at this magnification. It was classified as a "suspect" nodule, meaning that in time it probably would have become another hepatoma. An expanding trabecular tumor which has compressed the peripheral extranodular parenchymal cells is shown in figure 53.

The tumor shows widened muralia and increased basophilia of tumor cells. Aflatoxin B_1 was fed at 8 ppb for 12 months.

In figure 54 an advanced neoplasm is seen with adenomatous change and fibrosis in its center but in which the periphery remains trabecular in type. Aflatoxin B_1 was fed at 20 ppb for 12 months.

Figure 55 shows another advanced tumor with fibrotic change and with

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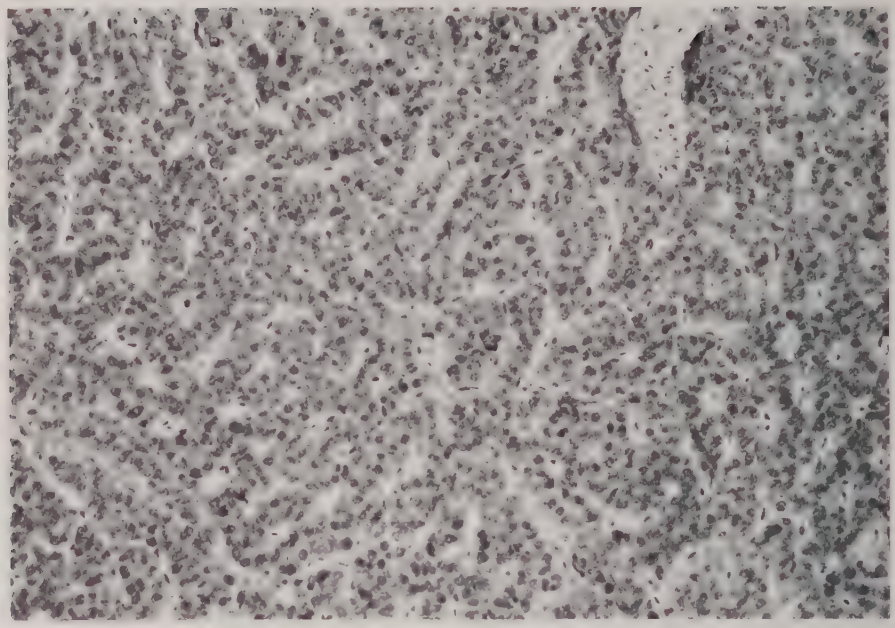

FIGURE 50.—Essentially normal rainbow trout liver showing muralia averaging two cells wide alternating with sinusoids and a vein filled with blood. × 190.

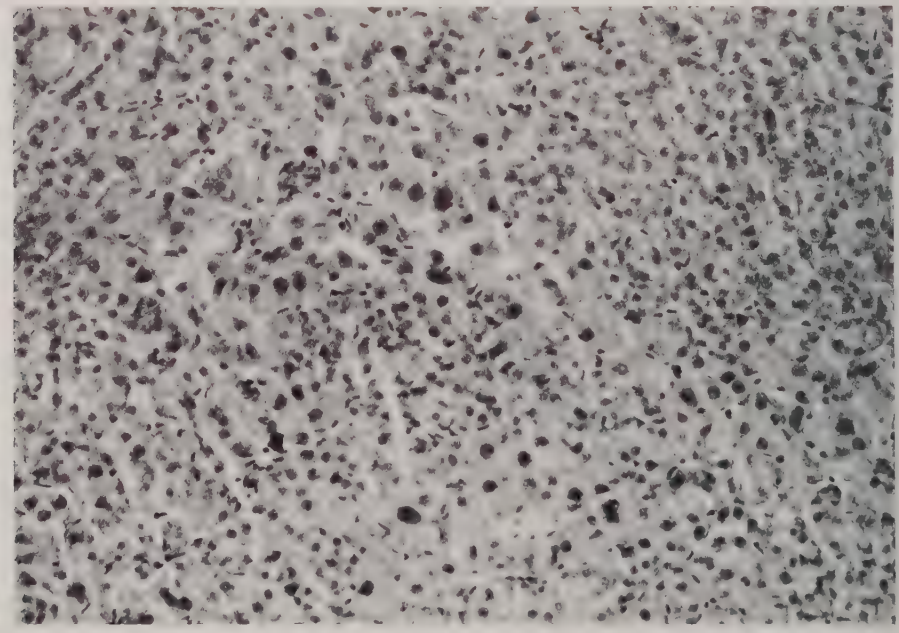

FIGURE 51.—Preneoplastic liver showing areas with increased nuclearity and areas of pale acidophilic cells some of which are swollen and some with bizarre basophilic nuclei (near center). Crude aflatoxin at 320 ppb for 9 months. × 190.

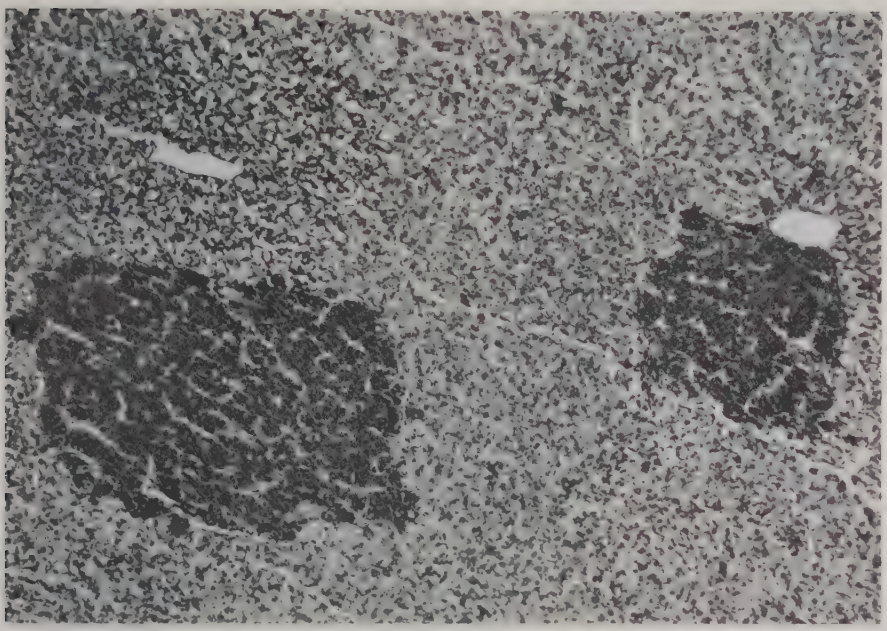

FIGURE 52.—Heavily vacuolated liver showing two early trabecular nodules. Crude aflatoxin at 80 ppb for 6 months. × 75.

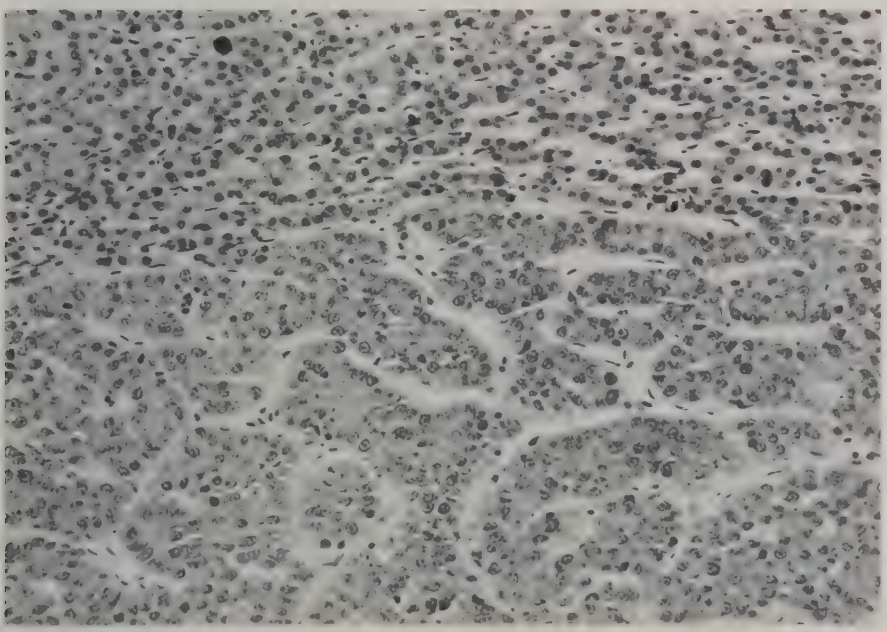

FIGURE 53.—Expanding trabecular hepatoma showing compression of peripheral parenchymal cells and widened muralia of the more basophilic tumor cells. Aflatoxin B_1 at 8 ppb for 12 months. × 190.

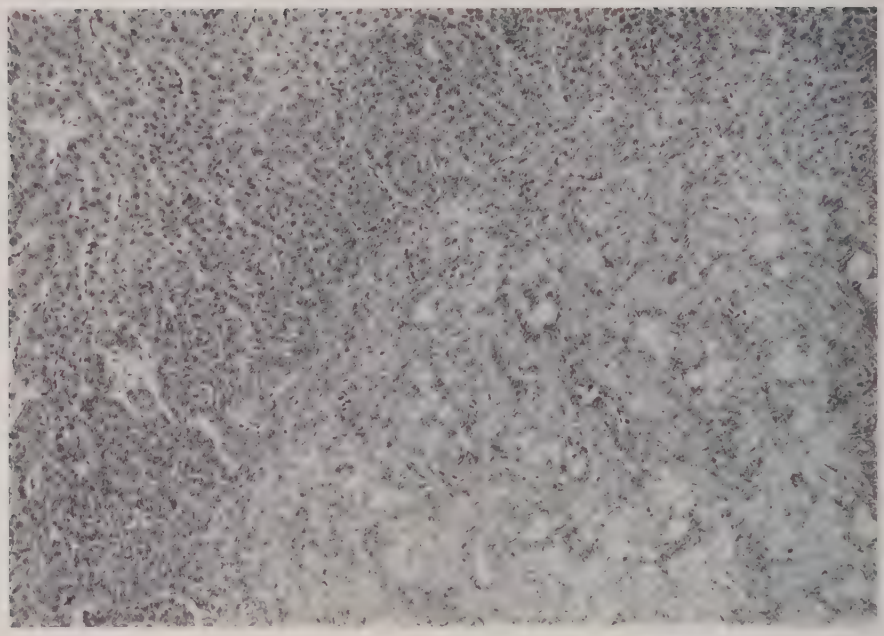

FIGURE 54.—Fibrotic and adenomatous change in center of advanced mixed tumor whose periphery is of the trabecular type. Aflatoxin B_1 at 20 ppb for 12 months. × 110.

somewhat anaplastic parenchyma cells, reduced vascularity, "degeneration clefts" (clear spaces), increased basophilia and widened muralia from a fish fed crude aflatoxin at 20 ppb for 12 months.

A small focus of proliferated cholangioles is apparent in figure 56 (bile ductules), in a toxic liver representative of an early change sometimes seen in trout livers after feeding massive doses of aflatoxins, especially at lower levels such as in this case where a total dose of 0.1 mg/kg body weight of aflatoxin B_1 was fed. This liver had minimal necrosis with extensive prenecrotic areas characteristic of a chronic response to aflatoxin.

A different liver from fish fed 320 ppb of crude aflatoxin in the daily diet for 6 months contained a prominent encapsulated bile duct proliferation or hyperplasia with some evidence of cystic change in some of the ducts. These exhibited the histology of typical bile ducts with typical columnar lining epithelium, lamina propria and muscularis outside of which was a distinct adventitial connective tissue layer much heavier than the delicate or very scanty fibrous film seen about the acinous-like groups of parenchymal cells characteristic of adenomatous change found in certain neoplastic livers.

Figure 57 shows an acute response to aflatoxin in the form of massive necrosis and hemorrhage of liver tissues but with partial sparing of tissues in portal zones. Crude aflatoxin was fed at 5 mg/kg b.w.

Three stages of necrosis are shown in figure 58; most advanced in the central area where all liver elements have died, intermediate in the upper portion where some evidence of life appears among tissue elements and least in the

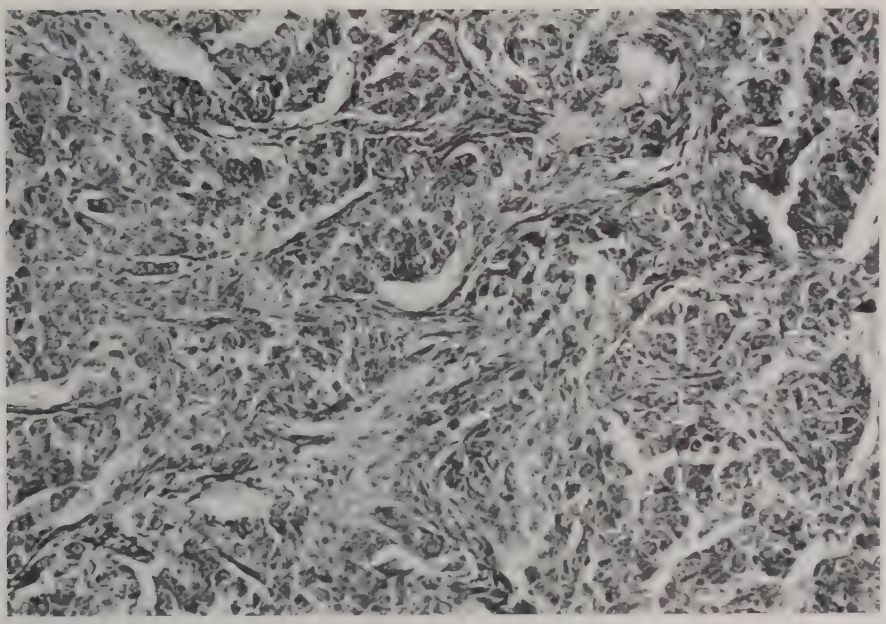

FIGURE 55.—Advanced hepatoma with fibrotic change showing somewhat anaplastic parenchymal cells, reduced vascularity, degenerative clefts (clear spaces), basophilia and widened muralia. Crude aflatoxin at 20 ppb for 12 months. × 190.

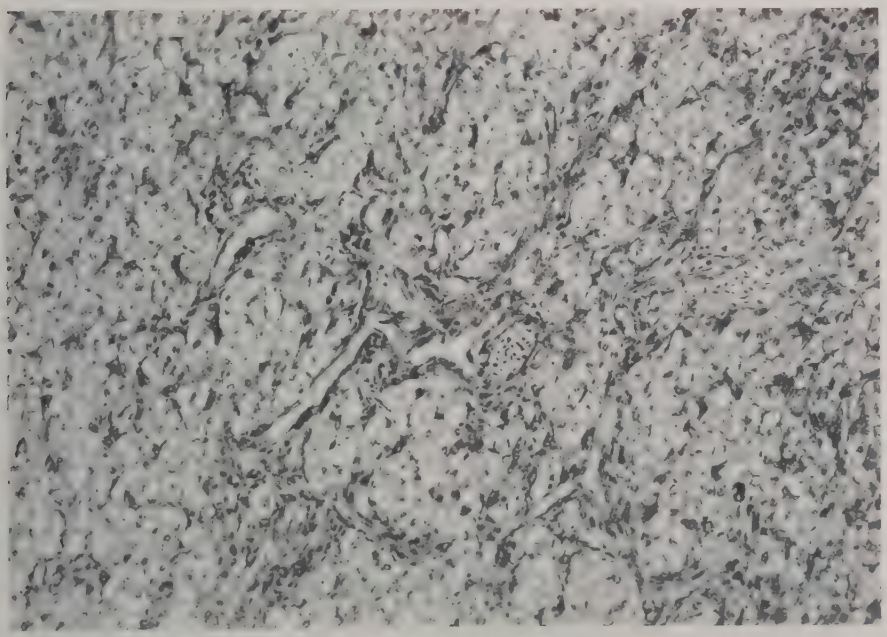

FIGURE 56.—Focus of bile ductule (cholangiolar) proliferation in toxic liver with large cytoplasmic vacuoles. Aflatoxin B_1 force fed at 0.1 mg/kg body weight single dose. × 110.

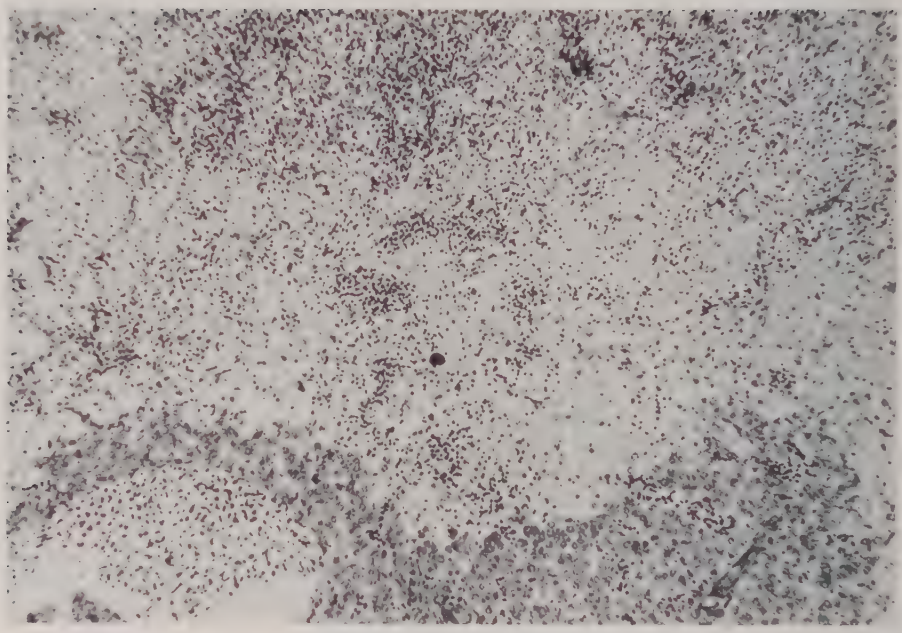

FIGURE 57.—Massive necrosis and hemorrhage of parenchymal cells and of other liver structures in central zones with sparing or parenchymal cells in portal zones. Crude aflatoxin at 5 mg/kg body weight. × 110.

FIGURE 58.—Three stages of massive necrosis, most advanced in center, intermediate above and least, below. Crude aflatoxin at 3 mg/kg body weight. × 190.

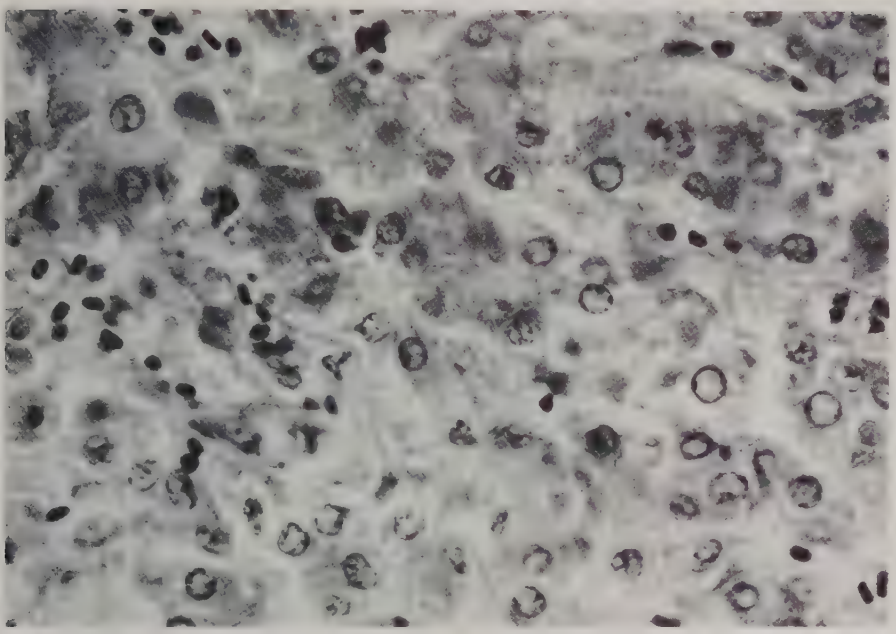

FIGURE 59.—Hepatic cytology of early necrosis showing chromatin margination of many and nuclear pycnosis of some parenchymal cells, mottling or scattered basophilia and stringy cytoplasm (center). Crude aflatoxin at 3 mg/kg body weight. × 625.

lower areas where tissues appear sick but are probably more or less functional. Crude aflatoxin was fed at 3 mg/kg b.w.

Some cytological details of early necrosis are evident in figure 59 with chromatin margination in many nuclei and some cells with pycnotic nuclei. Mottling with increased basophilia and with stringy cytoplasm in some of the reactive cells (center) may also be seen. Crude aflatoxin was fed at 3 mg/kg b.w.

Figure 60 shows more cytology of liver necrosis with many pycnotic nuclei and some parenchymal cells from which nuclei appear to have been dissolved or possibly extruded. Variable basophilia occurs in parenchymal cytoplasm which also reveals numerous small vacuoles some of which appear to be confluent. Pycnotic red blood cell nuclei, some of which appear rectangular in shape, are numerous in sinusoids and among desquamated parenchymal cells. Aflatoxin B_1 was fed at 1 mg/kg b.w.

Figure 61 shows relatively normal hemapoietic (head) kidney of rainbow trout.

A toxic head kidney with hyperemia is shown in figure 62 with swollen red blood cells, reduction of hemapoietic tissue, and increase in numbers of clear spaces suggestive of edema. Severe hydropic change and damage to hemapoietic and other tissues resulted when fish received repeated massive doses of aflatoxins and still survived from 10 to 21 days!

Figure 63 shows normal head kidney with three groups of pale adrenal cortical cells lining large blood sinuses peripheral to which are the more basophilic hemapoietic cells and sinusoids.

A toxic response found in figure 64 is similar to a stress reaction in head

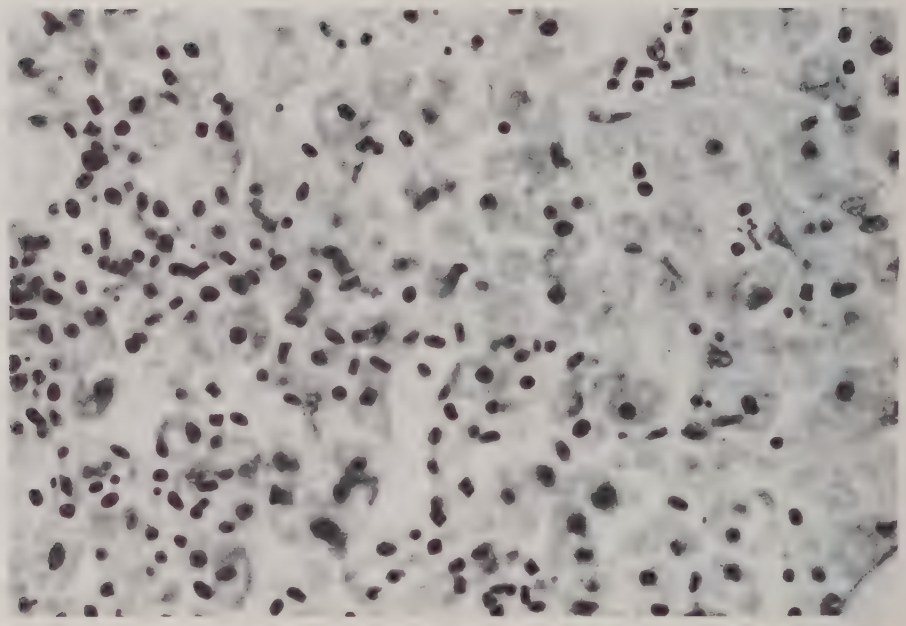

FIGURE 60.—Cytology of liver necrosis showing parenchymal cells with pycnotic nuclei and some from which nuclei appear to have been extruded or liquefied. Cytoplasm has variable basophilia with small discrete or confluent vacuoles. Pycnotic rbc nuclei are often rectangular as seen in sinusoids and among desquamated parenchymal cells. Aflatoxin B_1 at 1 mg/kg body weight × 1. × 625.

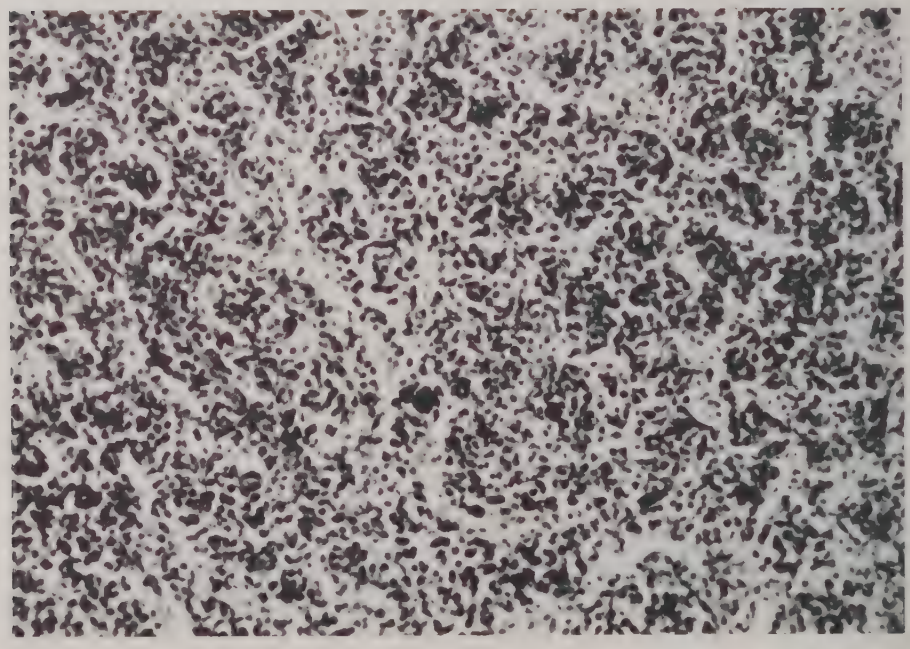

FIGURE 61.—Relatively normal head (hemapoietic) kidney of trout. × 190.

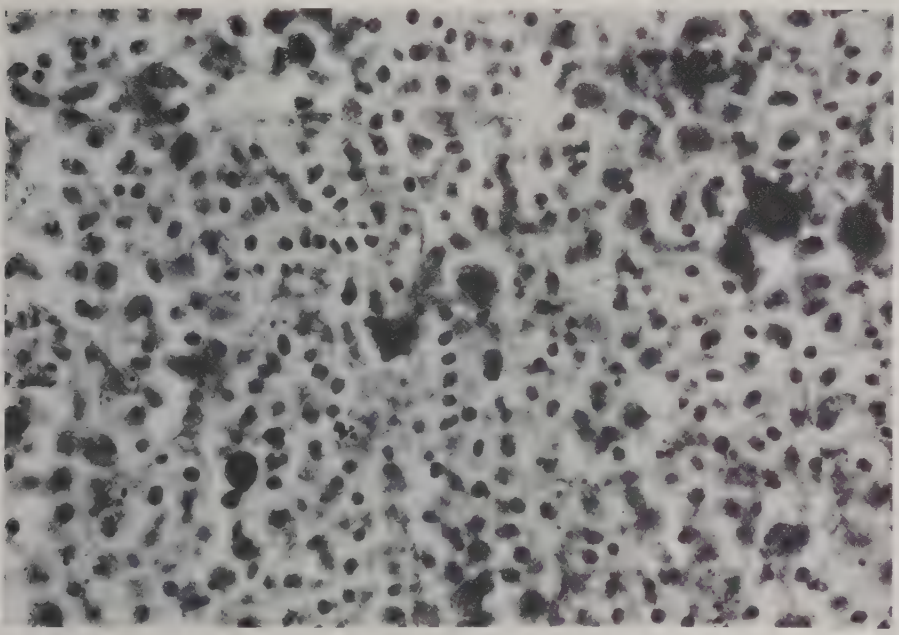

FIGURE 62.—Toxic head kidney showing hyperemia, swollen rbcs, reduction of hemapoietic tissues, and increase in numbers of clear spaces suggestive of edema. Crude aflatoxin at 3 mg/kg b.w. × 625.

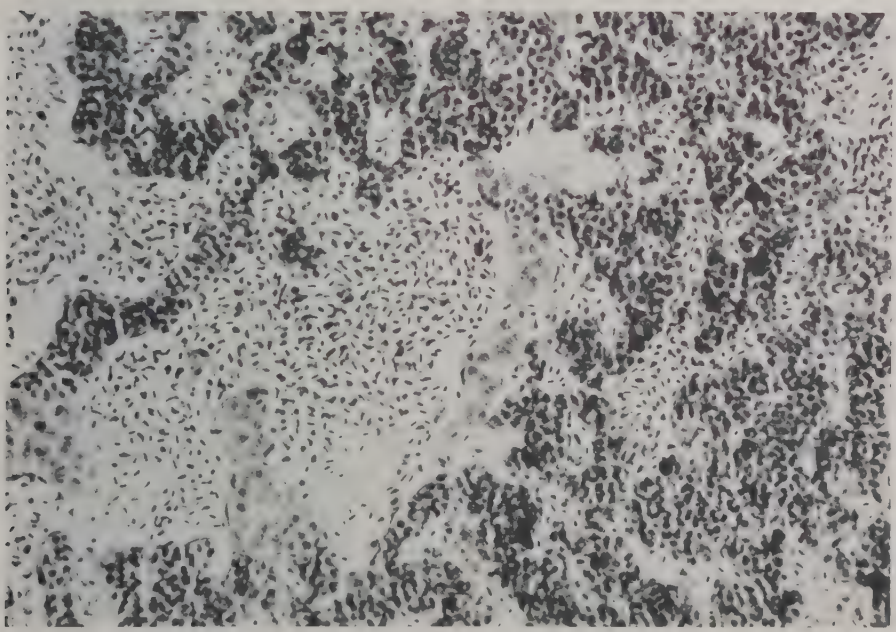

FIGURE 63.—Head kidney showing three groups of adrenal cortical cells adjacent to sinuses peripheral to which are the more basophilic hemapoietic cells and sinusoids. × 190.

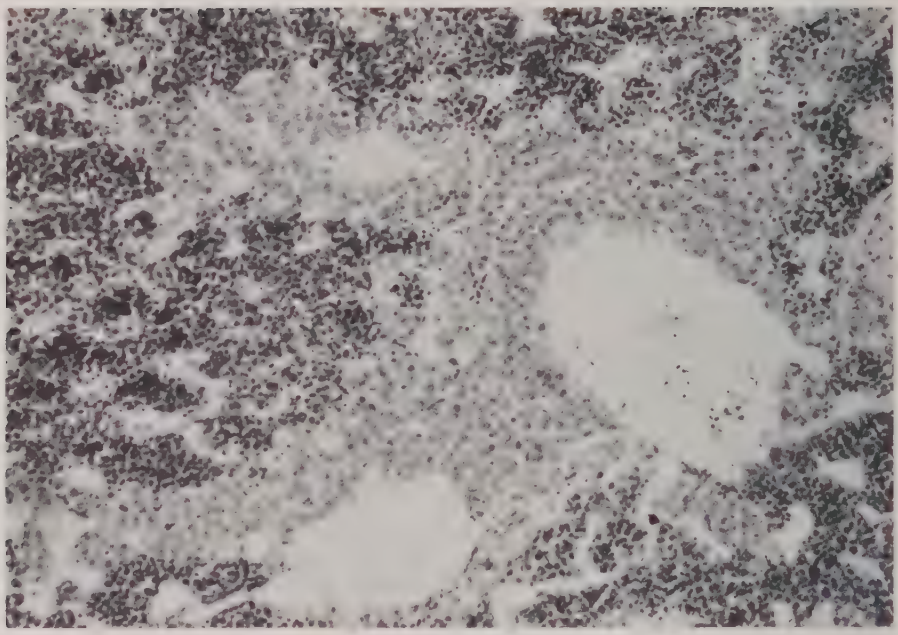

FIGURE 64.—Head kidney showing response to stress manifest by proliferation of adrenal cortical tissue peripheral to blood sinuses (pale cells). Crude aflatoxin at 3 mg/kg b.w. × 190.

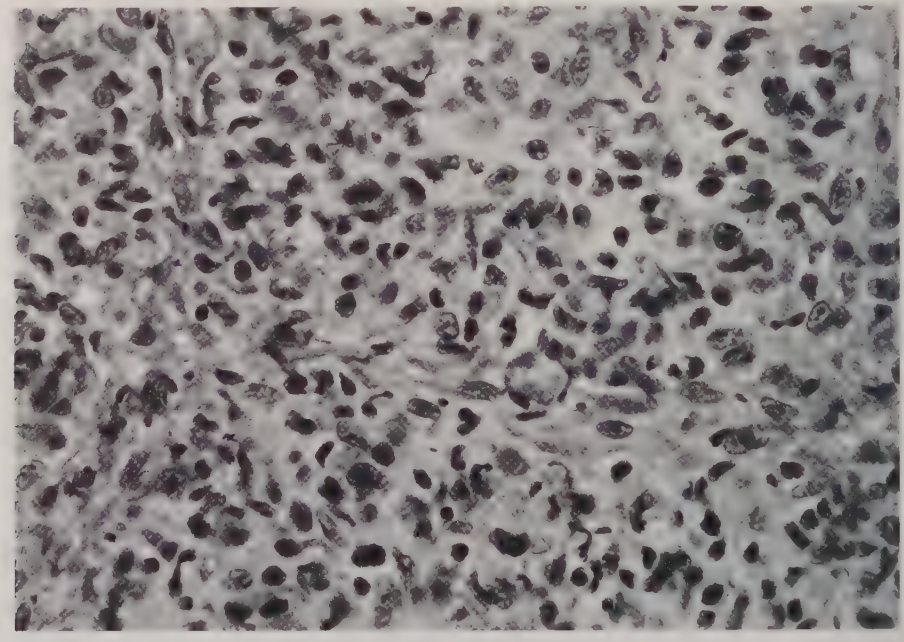

FIGURE 65.—Normal spleen of rainbow trout. × 625.

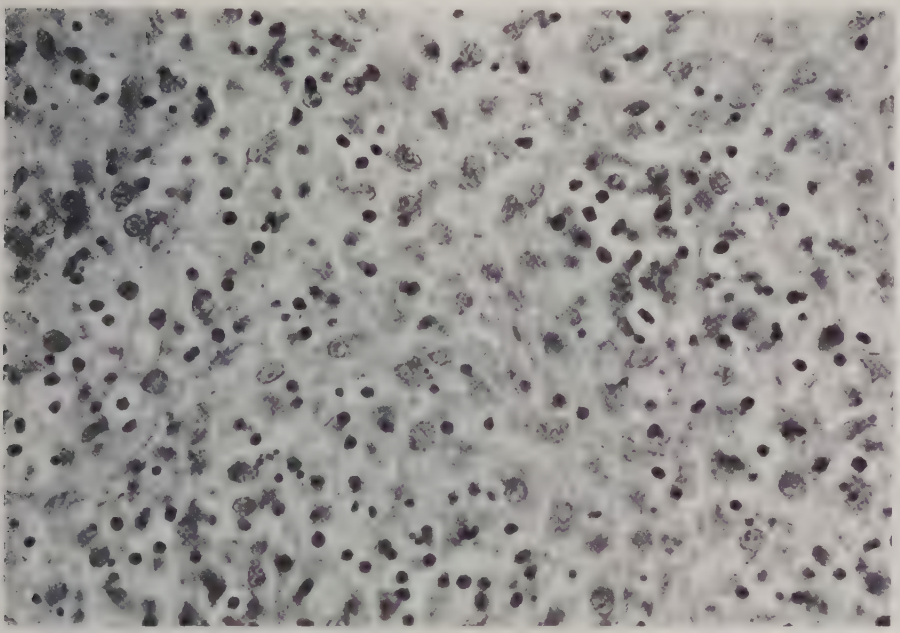

FIGURE 66.—Toxic spleen showing bizarre stroma cells and marked reduction of white pulp splenocytes. Crude aflatoxin at 3 mg/kg b.w. × 625.

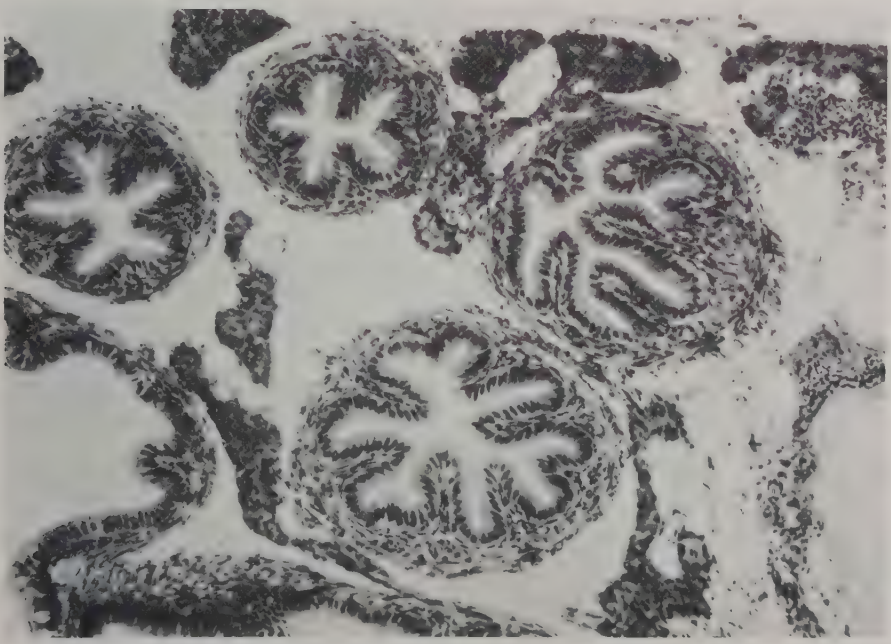

FIGURE 67.—Relatively normal caeca and pancreas of rainbow trout fingerling. × 110.

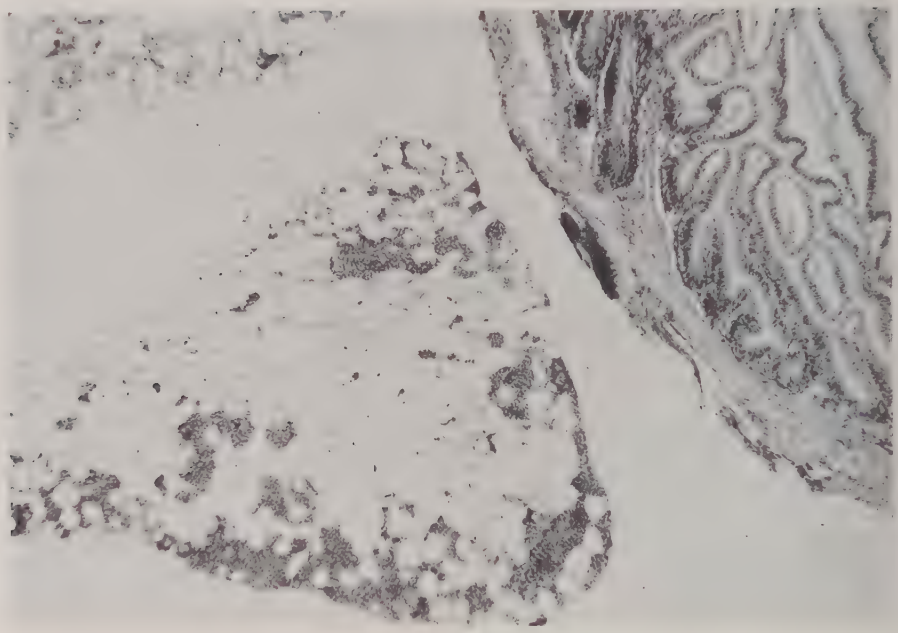

FIGURE 68.—Toxic caeca with slight edema and with hemorrhagic visceral adipose tissue. Crude aflatoxin at 5 mg/kg b.w. × 75.

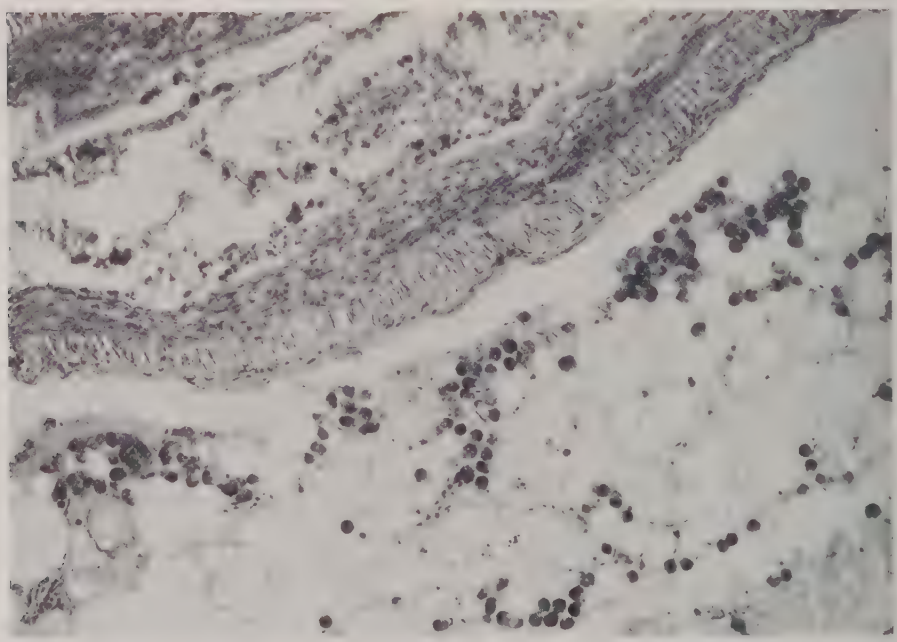

FIGURE 69.—Toxic caeca and pancreas showing sloughed mucosal elements in caecal lumen with edema of lamina propria and with swollen rounded and desquamated pancreatic acinar cells peripheral to visceral adipose tissue. Crude aflatoxin at 3 mg/kg b.w. × 190.

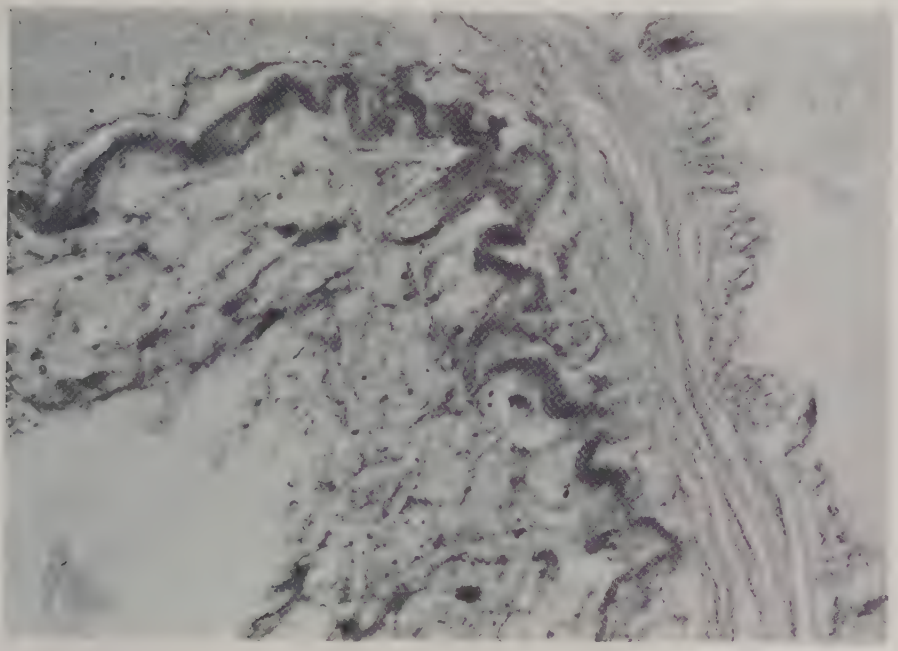

FIGURE 70.—Detail of edematous and partially necrotic pyloric caecum. Crude aflatoxin at 3 mg/kg b.w. × 625.

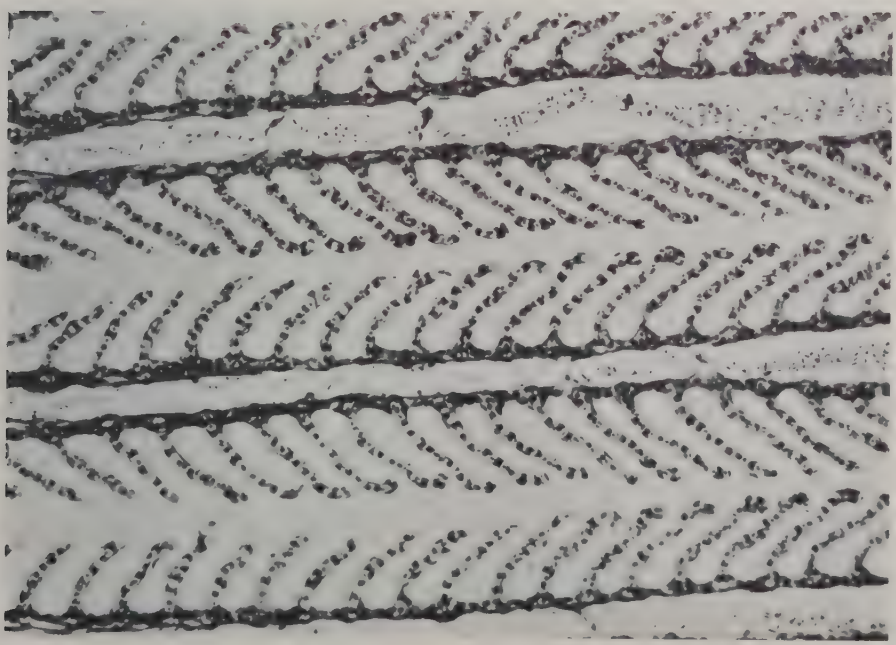

FIGURE 71.—Relatively normal gill filaments and gill lamellae of rainbow trout. × 110.

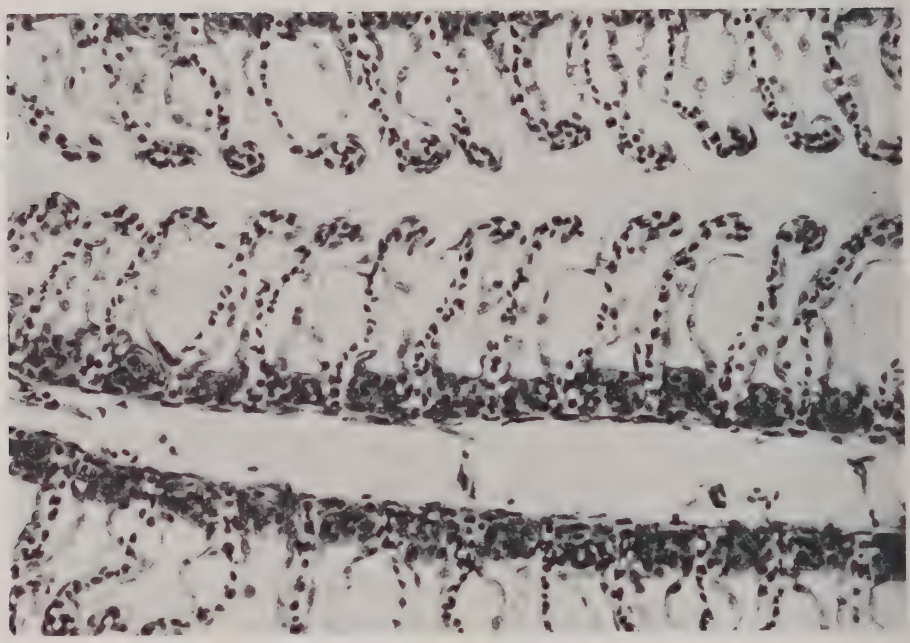

FIGURE 72.—Gill filaments showing hyperplasia of surface epithelium (basophilic) and extreme edema of some of the gill lamellae whose epithelia are in some places much distended from their central capillaries. Crude aflatoxin at 5 mg/kg b.w. × 110.

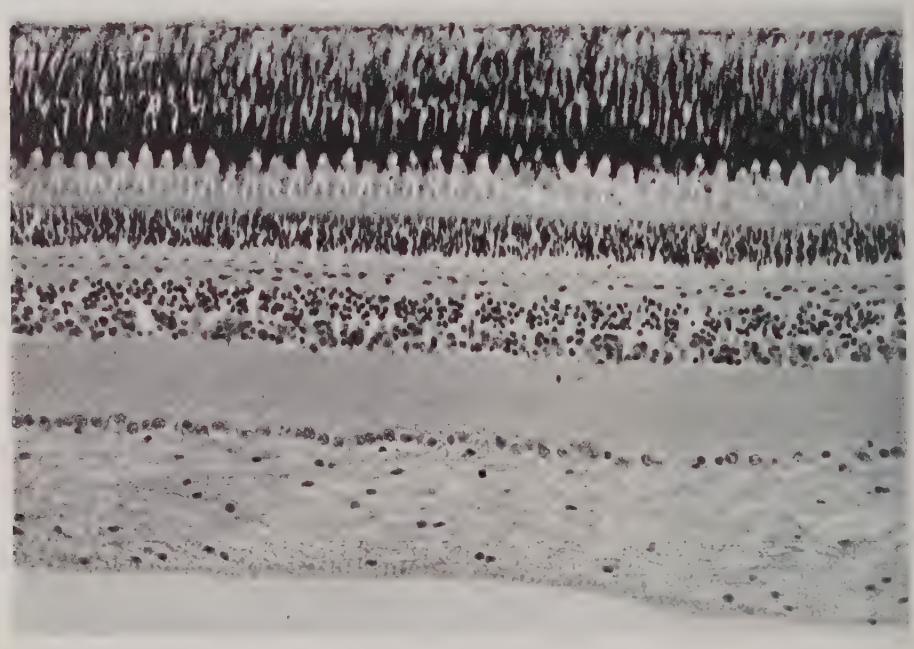

FIGURE 73.—Relatively normal retina of trout eye. × 112.

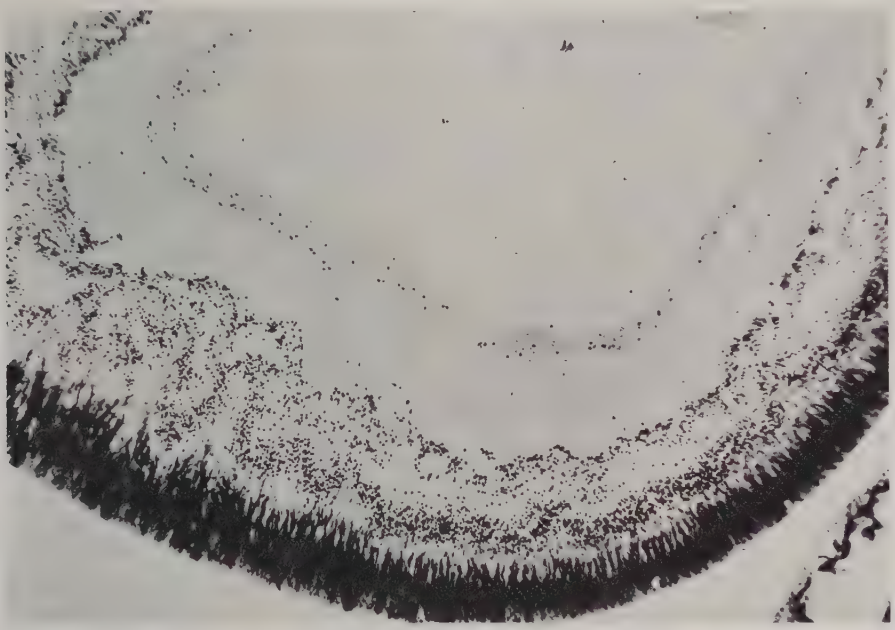

FIGURE 74.—Edematous retina with extensive disruption of all layers internal to layer of rods and cones external to which lies the heavy pigment layer. Crude aflatoxin at 5 mg/kg b.w. × 95.

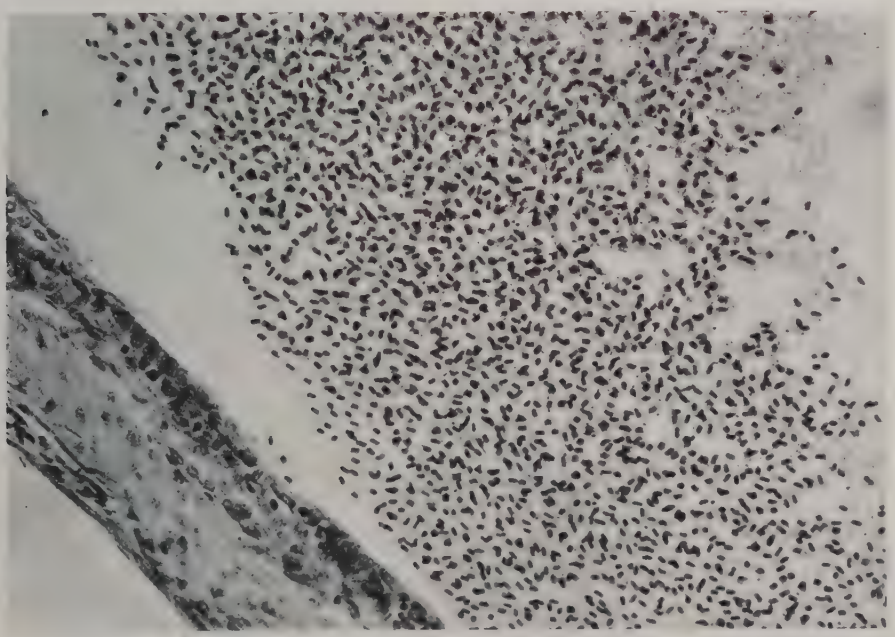

FIGURE 75.—Swim bladder of trout with heavy accumulation of blood in its lumen. Crude aflatoxin at 3 mg/kg b.w. × 190.

kidney which is manifest by proliferation of adrenal cortical tissue peripheral to blood sinuses (pale cells). Crude aflatoxin at 3 mg/kg b.w.

Figure 65 is relatively normal trout spleen.

Figure 66 is a toxic spleen with bizarre stroma cells and marked reduction of white pulp splenocytes. Crude aflatoxin at 3 mg/kg b.w.

In figure 67 we see relatively normal pyloric caeca interspersed with pancreas and taken from a rainbow trout fingerling as compared with figure 68 showing a toxic response in pyloric caeca showing mild edema and hemorrhage in the visceral fat. Crude aflatoxin at 5 mg/kg b.w.

In toxic caeca and pancreas, the caecal mucosa is largely sloughed into its lumen and the lamina propria is edematous while pancreatic acinar cells are swollen, rounded and desquamated (fig. 69). Crude aflatoxin at 3 mg/kg b.w.

In figure 70 a more highly magnified portion of the above caecum brings out details of hydropic response to aflatoxin including mucosal sloughing, cell necrosis, and edema evident particularly in lamina propria but to a lesser extent in the other coats as well. Crude aflatoxin at 3 mg/kg b.w.

Figure 71 shows relatively normal gill filaments and lamellae of a rainbow trout fingerling.

Hyperplasia of surface epithelium of gill filaments (basophilic tissues) and extreme edema of the lamellar epithelium of many gill lamellae are evident in figure 72. In some places lamellar epithelia are greatly distended from the central lamellar capillaries. Crude aflatoxin fed at 5 mg/kg b.w.

Figure 73 shows relatively normal retina from a fingerling rainbow trout.

Hydropic distension of retinal elements shown in figure 74 (cells and layers) is internal to layer of rods and cones, external to which lies the heavy pigment layer. Crude aflatoxin at 5 mg/kg b.w.

Figure 75 shows a swim bladder with blood-filled lumen in a trout with severe response to aflatoxin. Crude aflatoxin at 3 mg/kg b.w.

In some of the above fish there was also more or less focal necrosis of renal tubular epithelia and blue-grey amorphous casts were sometimes found in the kidney tubules. The identity of the casts remains undetermined.

ISOLATION, IDENTIFICATION, AND SOME BIOLOGICAL EFFECTS OF AFLATOXINS

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Aflatoxins are produced by several strains of *Aspergillus flavus* when the fungus is cultured experimentally on a wide variety of natural substrates, such as oilseeds, cereal grains, and certain animal products (e.g., whole milk powder). Recently [*1*] a synthetic liquid medium has been devised which supports aflatoxin production in yields nearly equal to those obtained from cultures on natural substrates. Since laboratory synthesis of the compounds has not yet been achieved, their production for various experimental purposes has been accomplished by the biosynthetic route.

In our laboratories, a potent toxin-producing strain of *A. flavus* (ATCC 15517) has been grown on wheat, or, more recently, on synthetic liquid medium. In the former instance, cultures were grown on 250 gm portions of coarsely ground sterilized wheat (brought to about 40 percent water content) contained in 3 liter Fernbach flasks fitted with cotton plugs. After inoculation with a spore suspension the flasks were incubated at 30° C. for 7 days. Yields of "crude aflatoxin" (see below) were in the order of 750 mg/kg dry weight of wheat.

For production on synthetic media, submerged cultures were grown in 3.5 liters of medium (1) contained in a 5-liter agitated jar fermentor. After inoculation, the cultures were incubated at 30° C. for 4 days, agitated at 600 rpm and aerated at a rate of 3 VVM. Studies of various medium compositions revealed that glucose, sucrose, or fructose are preferred carbon sources for aflatoxin production. Casamino acids are preferred as a nitrogen source, with ammonia being nearly as good. Zinc is required at levels of at least 0.4 mg/liter. Concentrations of aflatoxins obtained under these conditions were in the order of 40–60 mg/liter (expressed as aflatoxin B_1). Somewhat higher levels (60–80 mg/liter) were obtained in 100 ml shake-flasks.

In the isolation of aflatoxins from cultures prepared as above, extraction with chloroform has routinely been used. In the case of wheat cultures, the entire flask contents were extracted successively 3 times with 3 vols. chloroform in a Waring blendor (45 sec. at high speed, each extraction). Liquid cultures were filtered and the medium subjected to two successive liquid-liquid extractions with equal volumes of chloroform. Extraction was efficient, the partition coefficient being in the order of 600:1 into chloroform.

While these extraction procedures are suitable for isolation of aflatoxins in quantity from fungal cultures, they do not effect quantitative recovery of aflatoxins, particularly in the case of solid substrates. Therefore, in the extraction of meals, etc., for aflatoxin assay, other procedures have been devised by various workers. These have included

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blending with such solvents as absolute methanol, 55 percent aqueous methanol, 60:35:5 acetone:hexane:water and others. The consensus is that these solvents are more effective than chloroform in removing the last traces of aflatoxins present in various natural products.

A further advantage of chloroform extractions in preparative scale procedures is the relative stability of the aflatoxins in this solvent. Although the problem of stability has not yet been studied extensively or quantitatively, there have been several lines of evidence that degradation occurs in acetone or methanolic solutions particularly when exposed to air and light. Degradation also occurs when solutions spotted on thin-layer or paper chromatograms are exposed to UV or visible light and air. Dilute chloroform solutions appear to be relatively stable for long periods when stored in the dark at 5°C. or less.

In the isolation of aflatoxins from cultures, chloroform extracts are reduced to minimum volume after filtration. This concentrated solution is poured into a large excess of petroleum ether, and the aflatoxins precipitate as part of a flocculum which is recovered by filtration. This solid "crude aflatoxin" preparation contains typically 50–60 percent aflatoxins in the case of wheat cultures, and somewhat higher proportions in liquid media extracts. The relative proportions of the four aflatoxins present in this mixture varies with mold strains and culture media.

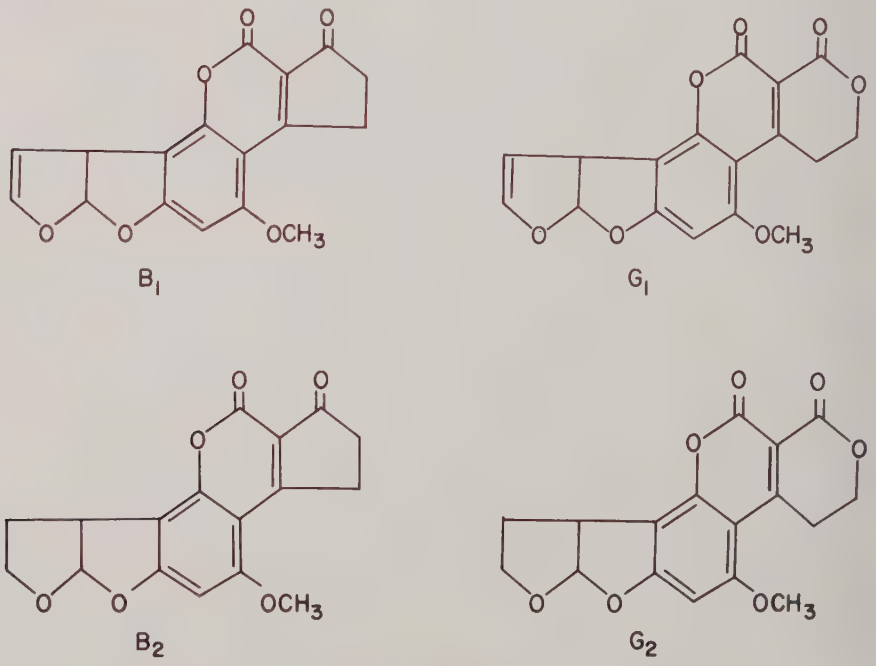


FIGURE 76.—Chemical structures of four aflatoxins.

TABLE 47.—*Summary of physical data on aflatoxins*

Aflatoxin	Molecular formula	MW	MP (°C)	a_D^{23}
B_1	$C_{17}H_{12}O_6$	312	268–269 (d)	−559
B_2	$C_{17}H_{14}O_6$	314	286–289 (d)	−492
G_1	$C_{17}H_{12}O_7$	328	244–246 (d)	−533
G_2	$C_{17}H_{14}O_7$	330	237–240 (d)	−473

In all cases, aflatoxin B_1 and G_1 predominate while B_2 and G_2 are present in small amounts.

The four aflatoxins in such mixtures can be fractionated chromatographically by several systems. Adequate resolution of the compounds is achieved on thin-layer chromatograms utilizing silica gel G HR (Merck) plates developed with 97:3 chloroform: methanol. The aflatoxins are detected by visual examination of the chromatogram under long-wave UV light in which they are intensely fluorescent. (It is possible to detect under these conditions in the order of 0.0005 microgram of aflatoxin B_1 in a single spot.) Aflatoxins B_1 and B_2 appear brilliant blue while G_1 and G_2 are yellowish green, with G_2 having blue overtones. Aflatoxin B_1 migrates with the highest R_f, followed successively by B_2, G_1, and G_2. Absolute R_f values are poorly reproducible, and normally a standard of known composition is run on each chromatogram for identification purposes. Somewhat better resolution of the individual components of the mixture can be achieved by partition chromatography on kieselguhr plates using 2:1 formamide: water as the stationary phase and benzene as the mobile phase [*2*]. Small amounts of individual aflatoxins have been isolated by preparative scale thin-layer chromatography on 0.75 mm thick plates. These conditions can be modified for column chromatography for larger scale fractionations.

Pertinent physical and chemical characteristics obtained in the course of identification and structure elucidation of the aflatoxins are summarized in tables 47 and 48. On the basis of these data as well as additional information from nuclear magnetic resonance spectroscopy and synthesis of several coumarin derivatives, structures for the four aflatoxins (fig. 76) have been proposed [*3,4*]. Additional evidence supporting the proposed structures has been derived from X-ray diffraction studies [*5*].

The four aflatoxins are the principal fluorescent components of chloroform extracts of *A. flavus* cultures grown on synthetic media. In extracts of wheat cultures, a complex pattern of fluorescent substances is typically seen on chromatograms. Certain of the non-aflatoxin substances probably represent components normally present in wheat. Others, present in relatively small amounts, are thought to represent degradation products (or intermediates in the biosynthesis) of aflatoxin. At present, none of these compounds has been identified chemically, and their possible structural relationship to aflatoxin awaits isolation and characterization. Structural similarities may be important determinants of their biological activity, since a range of potency has been shown among the four identified

TABLE 48.—*Summary of spectral data on aflatoxins*

Aflatoxin	UV absorption (ε)		IR absorption (CM^{-1})			$CHCl_3$ ν_{max}	Fluorescence emission (mμ)
	265 mμ	363 mμ					
B_1	13,400	21,800	1760	1684	1632	1598	425
B_2	9,200	14,700	1760	1685	1625	1600	425
G_1	10,000	16,100	1760	1695	1630	1595	450
G_2	11,200	19,300	1760	1694	1627	1597	450

aflatoxins [6]. These relationships between structure and acute lethality in ducklings is shown in table 49, in which the maximum ratio of potency between B_1 and G_2 is approximately 8:1.

The precise structural configurations responsible for aflatoxin-like activity are not known. However, there have been several observations of at least partial retention of potency in aflatoxin derivatives. For example, the reduction product of aflatoxin B_1 (tetrahydrodesoxo-aflatoxin B_1) causes detectable bile duct hyperplasia in ducklings, the required dose being approximately 100-fold greater than that for B_1 [4]. The isolation of a metabolite presumably derived from aflatoxin from milk of cattle fed the compound has recently been reported [7]. This compound, not yet identified chemically, has fluorescence properties similar to those of B_1, but migrates on thin-layer chromatograms with a greatly reduced R_f value. This compound also causes bile duct lesions similar to those caused by B_1 but with somewhat reduced potency.

The structural configurations necessary for toxic manifestations of the aflatoxins have also been studied with seven intermediates in the laboratory synthesis of aflatoxin (table 50). These compounds were administered orally according to the 5-day schedule previously described for aflatoxin B_1 bioassay [8]. Preliminary experiments indicated that none of the compounds studied had lethal effects at doses representing 100, 50, or 10 times the dose of aflatoxin required to induce detectable bile duct change. The livers of these animals were examined histologically in order to determine whether any of the compounds have low potency of the type shown by aflatoxin B_1.

TABLE 49.—*Acute (6-day) oral toxicity of aflatoxins to ducklings**

Aflatoxin	LD_{50}**	Ratio of activity
B_1	18.2	$B_1:G_1=2.15$
B_2	84.8	$B_2:G_2=2.03$
G_1	39.2	$B_1:B_2=4.66$
G_2	172.5	$G_1:G_2=4.40$

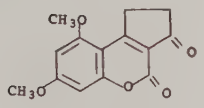
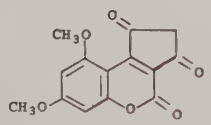
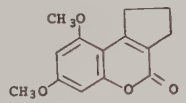
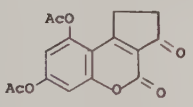
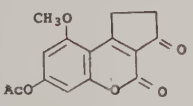
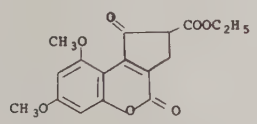
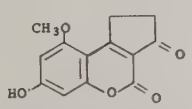
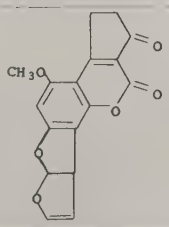
* Data of Carnaghan, Hartley, and O'Kelly, Nature 200:1101, 1963.
** Micrograms/50 grams body weight.

The growth rate, body weight, and histologic data (bile duct hyperplasia score) are summarized in table 50. Each value presented is the mean of five animals per group. It can be seen that none of the synthetic compounds had significant effects on growth or liver weight with the possible exceptions of compounds 6 and 7. None of the differences among these data are statistically significant because of the great variability among individual animals characteristic of duckling growth patterns.

None of the test compounds induced detectable bile duct hyperplasia or other histologic changes at any dose level. Reproducibility of our earlier observations (8) concerning the sensitivity of the bile duct endpoint in detecting aflatoxin B_1 activity is illustrated by the response to these compounds in table 50. A daily dose of 0.5 micrograms results in minimal lesions, and 3.0 micrograms per day caused moderate lesions, slight growth depression, and decreased liver size. It must be concluded that these intermediate compounds have no demonstrable toxic properties under the conditions of this experiment. The highest dose level used (500 micrograms) represents a total dose amounting to 25 times the LD_{50} of aflatoxin B_1 (approximately 20 micrograms in animals of this age and weight) or a daily dose of 5 times the LD_{50}.

There was a suggestion of growth suppression with compounds 6 and 7. It is interesting to note that the latter compound is structurally most closely related to aflatoxin B_1. Further studies will be required to determine whether any of these compounds prove to be

TABLE 50

Compound No.	Structure	Total dose (μg)	Body wt. (gms)	Liver wt. (% b. wt.)	BDHS
Control	H_2O	0.25 ml	86	6.90	0
Control	DMSO	0.25 ml	96	7.53	0
1		50	81	7.36	0
		250	98	8.18	0
		500	101	7.41	0
2		50	96	7.54	0
		250	81	7.90	0
		500	110	6.96	0
3		50	86	7.89	0
		250	105	6.19	0
		500	121	6.23	0
4		50	106	5.97	0
		250	96	6.07	0
		500	92	6.60	0
5		50	92	8.06	0
		250	79	7.10	0
		500	85	7.13	0
6		50	107	8.01	0
		250	96	8.28	0
		500	64	7.33	0
7		50	80	6.65	0
		250	84	6.93	0
		500	71	6.49	0
Aflatoxin B_1		2.5	74	7.28	4.0
		15.0	62	6.35	12.0

carcinogenic and also whether the acute toxic manifestations are necessarily related to the chronic carcinogenic process.

Aflatoxin B_1 as well as mixtures of aflatoxins containing this compound are known to be hepatocarcinogens. It remains to be determined, however, whether structure-activity relationships of the type obtained in acute or subacute toxicity studies will also be associated with carcinogenic potency. Investigations into structural requirements for carcinogenic activity will be facilitated by increasing availability of purified aflatoxins and structurally related compounds.

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Discussion

DR. WOGAN: In keeping with the tradition that has been set by several previous speakers, many people at M.I.T. have been collaborating in one or another aspect of the aflatoxin research program.

This whole thing actually has some of the characteristics of these tumors that we have been talking about in its pervasive nature. Starting with a nuclear and a very modest effort in my own group, it has come to involve a number of people, not only in the department from which I come, but also in the department of chemistry. The structure characterization was actually done largely by Professor Buchi and Dr. Asao on material which we had isolated and provided for the chemical elucidation.

I would also like to mention particularly Professor Mateles, who is a biochemical engineer interested in the biosynthesis of aflatoxins by *A. flavus,* and has provided us with some very valuable material, not the least of which is that he worked out a good liquid culture medium in which quite good yields of aflatoxins could be obtained. Also a group at Hoffman-La Roche was kind enough to run a large scale pilot plant scale fermentation and provided us with a great deal of useful aflatoxin concentrate.

I would like to mention a hepatoma incidence in rats fed aflatoxin contaminated diets for periods of 294 to 384 days. These data were obtained actually from samples of peanut meal which Professor Newberne had studied earlier and which we assayed for aflatoxin content.

There was very good correlation in rats between hepatoma incidence and the aflatoxin concentration in the diet, levels of one part per million caus-

ing in the order of 80 percent tumor incidence.

Since I received Dr. Halver's data on trout, I took the liberty to make a similar plot. There is a semilog plot tumor incidence with aflatoxin B_1 and G_1, and it turns out that the slope of the trout response is very much flatter than the slope of the rat response. While these are very preliminary data, I think it might indicate the reason that higher tumor incidences are not found, even at very high aflatoxin concentrations in the diet.

We now have a large number of rats to test this dose response relationship. We are currently feeding 1 part per million, 300 parts per billion, and 30 parts per billion. These animals have not been on test long enough yet to give us any usable data.

One point that I want to make is the presence of a red pigment which overlays G_1 and G_2 in chromatograms of wheat cultures, and the activity of which is unknown at the moment.

The other point of interest is the presence below G_1 and G_2 of one or two blue fluorescent bands, which have the same mobility characteristics as the so-called milk toxin that the Dutch workers have isolated from milk from cows fed aflatoxin-containing diets.

We have looked at the toxicity of this compound at very low levels and found it not very active, or not active at the levels we used, but this is not conclusive evidence that this is not a natural product or natural component of aflatoxin mixtures.

The point of this whole discussion is that the mold probably produces a spectrum of fluorescent compounds which may be structurally related to the aflatoxins, or, to bring up another point, may be degradation products formed—or, be artifacts formed during the handling of these crude mixtures. As Dr. Falk will point out in more detail, the aflatoxins are not particularly stable under many conditions.

We looked in rats at some of the acute effects of aflatoxin administration on various aspects of liver metabolism. One of the principal findings, which we consider to be of primary interest is the effect on the incorporation of leucine, C-14 labelled, into total liver proteins.

During the first 12 hours there was a very profound expression on protein synthesis. This agrees with earlier findings in duck liver slices and in other systems. If one follows this long enough, one sees a secondary stimulation, the meaning of which is not now clear. As pointed out earlier, this may represent regeneration of damaged liver cells.

We determined then to look in more detail at this early suppression and we chose as our model system the inducibility of several enzymes involved in amino acid metabolism, among which are tryptophan pyrollase and tyrosine transaminase.

We have found that aflatoxin suppresses the basal level and also the inducibility of tryptophan pyrollase by hydrocortisone. We have studied this in some detail and have come to the conclusion that nucleic acid metabolism is being affected in some way by aflatoxin, and we looked forward the possibility of trying to pin this down to a specific site.

We were able to produce C-14 labelled aflatoxin, by feeding the mold C-14 label methionine. It selectively takes the methyl group from methionine and puts it in this position. We have accumulated enough of this material at high enough specific activities to be able to use it in rats in successful experiments.

We have used two types of labelled material and find that when the methyl labeled aflatoxin is given to weanling

rats in a single dose, they excrete about 70 percent of this label in 1 day (24 hours), about 25 percent of this is as CO_2, indicating that demethylation occurs fairly readily. On the other hand, with the acetate labelled material, something on the order of seven-tenth of one percent of the activity comes out as CO_2 and the remainder is excreted in feces and urine. The total excretion appears to be about the same in both instances.

We hope in the near future to have available a derivative of this compound for the structure activity relationship which has the oxygen missing and has two rings open.

MR. SINNHUBER: Can you comment on Dickens' work regarding the 3–4 position?

DR. WOGAN: His work would indicate that the lactone configuration is active only when it is a gamma lactone —that the activity is abolished by the removal of this double bond. Unfortunately we have not succeeded in removing this double bond without destroying a great many other functionalities.

DR. SIMMONS: If the lactone is split with alkali is the corresponding compound active or is it reactivated in the gut?

DR. WOGAN: I don't know that anyone has actually opened the lactone ring, isolated the compound and studied its toxicity.

DR. BUHLER: On your work on leucine incorporation were the animals sacrificed at the same time of day each day? I am sure that you are aware that there is a pronounced diurnal variation in amino acid incorporation in regenerating liver.

DR. WOGAN: We didn't find it in the controls, which were sacrificed at the same time. The other point that I should have mentioned in that same connection was that subsequently there are a number of other questions that could be asked about that experiment. We repeated this in an *in vivo-in vitro* experiment in which the animals were administered the aflatoxin and then killed at intervals and the incorporation done *in vitro* rather than in administration to the animal. This resulted in essentially the same curve with a different axis.

DR. LA ROCHE: Has this variation in incorporation of amino acid been verified in fish?

DR. WOGAN: Not to my knowledge.

DR. KUMMEROW: How about the effect on lipid metabolism?

DR. WOGAN: We did preliminary studies and the results are a little ambiguous. The rat does not rapidly start accumulating lipid after a single administration. It does over a period of 2 weeks increase by perhaps 3 percent. This would be about a 50 or 60 percent increase. However, the rate of incorporation of precursors into the liver lipids is affected to a fairly great extent. That is, while the total concentration doesn't change remarkably over this period that we studied, there are obviously changes in turnover.

A single dose of aflatoxin at the level of three milligrams per kilo virtually renders tryptophan pyrollase uninducible by hydrocortisone for periods up to 20 days.

On the other hand, with tryptophan as the inducer the induction occurs at a rate of about 50 percent to 60 percent of normal. The effect here, presumably, is one of stabilization of existing enzymes, a suppression of degradation rather than a true induction.

On shorter term experiments in which we followed the 12-hour induction curve of tryptophan pyrollase the results qualitatively looked very much like actomy-

osin D; that is, a suppression if the aflatoxin is given during the first three hours, but insensitivity beyond the third hour. This is the basis on which I said that we thought nucleic acid metabolism was involved.

DR. NOVELLI: Did you see the same pictures with tyrosine transaminase?

DR. WOGAN: The same pattern exists with that enzyme, yes, and also with fructose 1–6 and also with glucose 6-phosphatase.

BIOLOGICAL ACTIVITY OF THE AFLATOXINS IN DOMESTIC AND LABORATORY ANIMALS

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Abstract.—Reports from field outbreaks and from laboratory experiments have shown some notable differences as well as similarities among species in regard to the hepatotoxic effects of aflatoxin. The induction of malignant hepatocarcinomas, now adequately confirmed in several laboratories, points up the potential seriousness of the problem. We now feel that there is adequate experimental evidence available to link earlier field outbreaks of certain noninfectious diseases in domestic animals and dogs to aflatoxin and that the choline-deficiency tumor of rats reported earlier was complicated by mold toxins. The chemical assay combined with the duckling bioassay provide a means of permitting the continued use of the valuable peanut meal protein by screening aflatoxin from the edible foodstuffs destined for human consumption and livestock feeds.

During the period 1945–53, practicing veterinarians and veterinary diagnostic laboratories in the Southeastern United States encountered sporadic, recurring outbreaks of a non-infectious disease in swine, cattle and dogs. The disease in hunting dog kennels was especially disastrous. This served to initiate an intensive investigation into the cause of the malady, but results of these studies left many questions unanswered [*1,2,3*]. The disease in swine and cattle was associated with moldy feeds, more often badly damaged corn; in all documented outbreaks in dogs the commercially prepared feed reportedly contained peanut meal as a source of dietary protein. Little of a definitive nature other than morphologic characteristics of the disease was learned beyond these facts.

A number of veterinary diagnostic laboratories in this country had observed occasional isolated outbreaks of a disease in turkeys with characteristic histopathologic changes in the liver not attributable to previously diagnosed conditions. My first experience with the disease in turkeys was at the University of Missouri Veterinary Diagnostic Laboratory during the 1955–57 period. Gross and microscopic lesions observed in the few cases presented to our laboratory were identical to those described more recently in Britain [*4*]. In addition, my first encounter with trout hepatoma occurred during this same period when several trout from the hatcheries in Southeast Missouri were presented to our laboratories with typical liver tumors. Our service did not get involved beyond the morphologic diagnosis but it was noted that the hatchery fish were fed a commercial chow which contained cottonseed meal as a source of part of the protein.

In the midforties, a diet for laboratory animals was formulated in England [*5*] (MRC #18) which contained peanut meal as a source of part of the protein. This diet was associated by Paget [*6*] with a noninfectious disease in guinea pigs, characterized by gross subcutaneous edema, ascites and hepat-

ic changes. Stalker and McLean [*7*] reported another outbreak of the disease in guinea pigs which occurred in 1956 with lesions identical to those described by Paget. Stalker and McLean agreed with Paget that the source of trouble was a toxic factor or a deficiency state associated with some batches of the formulated MRC #18 diet.

In 1957, abortions and deaths in a guinea pig colony [*8*] at the Veterinary Laboratory at Weybridge, England, was associated with unknown dietary factor(s). Losses ceased when the diet was changed. Subcutaneous edema and ascites were constant findings at necropsy. Later, Paterson et al. [*9*] demonstrated that this disease was attributable to toxic peanut meal included in the diet.

During the years from 1946 to 1955 a series of papers [*10,11*] from Auburn University reported the occurrence of liver tumors and other neoplasms in rats and chickens fed choline-deficient diets. Peanut meal was used as a major source of dietary protein in these experiments. Subsequent to 1955 it was reported [*12*] that there had been a sharp decrease in the incidence of tumors observed in experimental animals fed peanut meal diets. These observations coincided with the more efficient methanol extraction methods used in the preparation of peanut meal used in the diets. A few years later [*13*] a very high incidence of liver cell tumors was observed in rats fed diets containing unextracted rather than methanol-extracted peanut meal. This observation together with the finding a year later [*14*] that even exhaustive methanol extraction left traces of aflatoxin in contaminated peanut meal raised important questions about the nature of choline-deficiency liver tumors in rats.

The outbreak of a disease in turkeys in England in 1960 [*15*] served to emphasize the seriousness of the toxicological problem facing the livestock industry as well as other segments of the economy involved in the food chain. The epizootic in turkeys initiated investigations which led to the isolation and characterization of a class of toxic mold metabolites, the potential hazard of which is only now beginning to be generally recognized. The initial problem in turkeys and ducklings [*16*], pigs [*17*] and calves [*18*] was traced to peanut meal included in the diet of the animals. The brilliant investigative efforts of a number of laboratories have revealed that the biological activity contained in the peanut meal centered around a group of toxins elaborated by some strains of *Aspergillus flavus* mold cultures. The chemical structure of the major fractions of the toxins are now well known [*18*] and the biological activity of the combined fractions as well as some of the purified [*14*] single crystalline fractions have been studied in a number of species.

Before the identity of the toxic principle had been established, however, the effects of contaminated peanut meal were studied in laboratory animals and had been reported in the major species of domestic animals [*15,16,17,21*]. Following the identification of the toxic principle and with the crude material tested in several species, it has been established that symptoms and lesions are virtually the same with the isolated chemical complex as with contaminated peanut meal. Results of these investigations form the basis for this presentation.

In most species, symptoms of toxicity have not been very specific. The first clinical observations in all species usually include inappetance and reduced growth rate in the young, or loss of condition in mature animals; dosage level will modify the symptoms, however, apart from signs of unthriftiness, there

are usually no clear indications of serious disease until a few days (2–4) before death. At this point, several nonspecific but characteristic symptoms may be exhibited. These include ataxia, convulsions and opisthotonus at death in ducklings; ataxia, circling, severe tenesmus, rectal eversion, ear twitching and grinding of teeth in calves; general malaise and weight depression in rats.

There is considerable variation in susceptibility among the different species to the effects of aflatoxin. Young animals are more sensitive than mature ones. Ducklings are particularly susceptible with turkeys, pheasants, and chickens somewhat less sensitive. Larger farm animals vary in their response with young pigs (4–8 wks), pregnant sows and calves up to 6 months of age most adversely affected in field outbreaks [*20*]. In our laboratories only one series of weanling pigs have been exposed to aflatoxin B_1 under controlled conditions. The dose was 2.0 mg/kg body weight orally and 5/6 succumbed in 18 hours; the remaining pig survived an additional 24 hours. In such acute response the only significant lesions are severe fatty liver and widespread petechiae. Three- and four-year-old heifers have become clinically ill after eating contaminated peanut meal as a portion of the diet for 7 months, while 8- to 10-year-old cattle required 12–14 months feeding of toxic meal before adverse effects were noted. No effect was observed on conception, gestation or parturition in any of the cattle and calves born to these cows have developed normally for at least a year. Milk yield was significantly reduced, however, in cows fed contaminated feeds.

Of the domestic species studied, sheep are most resistant to aflatoxin and no field outbreaks in this species have been reported. Dogs appear to be quite sensitive to aflatoxin in acute studies, but reports of chronic studies are not available.

Several species of laboratory animals have been tested for susceptibility to the effects of aflatoxin. Guinea pigs and ferrets are most sensitive of the usual laboratory species tested [*20*], rabbits are less so, and rats are comparatively resistant [*14,19*]. Mice appear to be very resistant; however, adequate data on a number of different strains are lacking [*20,22*].

Table 51 lists comparative pathology of several species of animals. Of the species studied, ducklings are the most sensitive to effects of aflatoxin. It will be noted in table 51 that the single lesion common to all species studied is a proliferation of bile duct cells. The only exception is the mouse and this is based on only one study, a long-term one, with contaminated peanut meal conducted in our laboratory. Detailed acute or sub-

TABLE 51.—*Comparative pathology in various animal species fed aflatoxin-contaminated feed or purified aflatoxin*

[Minus sign indicates negative results, a zero indicates unknown]

Liver lesions	Calves	Cattle	Swine	Sheep	Dog	Duckling	Adult duck	Poult	Chick	Rabbit	Guinea pig	Cat	Rat	Mouse
Acute necrosis and hemorrhage	−	−	+	−	+	+	−	+	−	+	+	+	+	−
Chronic fibrosis	+	+	+	0	+	±	+	−	−	0	−	0	+	−
Regeneration nodules	−	+	+	0	+	+	+	+	−	0	+	0	+	−
Bile duct hyperplasia	+	+	+	0	+	+	+	+	±	+	+	+	+	−
Veno-occlusive disease	+	+	−	0	−	−	−	−	−	0	−	0	−	−
Enlarged hepatic cells	+	+	+	0	+	+	+	+	−	+	+	0	+	±
Liver tumors	0	0	0	0	0	−	+	0	0	0	0	0	+	+

TABLE 52.—*Effects of extracts of* Aspergillus flavus *cultures on weight gains and bile duct hyperplasia of ducklings*
[From Newberne, Carlton, and Wogan, 1964, abridged]

Group No.	Treatment	Duration (days)	Final total body wt. (gm)	Liver wt. (gm)	Per cent body wt.	Bile duct hyperplasia[1]
1	None	7	170	11.8	6.3	0.0
2	Chloroform extract of *A. flavus*	7	86	3.6	4.1	34.0
3	Petroleum ether extract of *A. flavus*	7	159	10.8	6.8	0.0

[1] Graded 0 to 4+, multiplied by ten and averaged.

acute studies may indicate a different response in this species. Additional important observations, illustrated in table 51, include the apparent complete resistance of sheep and the veno-occlusive response in the bovine to the effects of aflatoxin. Liver tumor induction may be a function of time in species now listed as negative or unknown.

Table 52 lists the effects on ducklings of a chloroform extract and of an ether extract of an *Aspergillus flavus* culture.

TABLE 53.—*Bile duct hyperplasia in ducklings fed peanut meal for short periods of time and liver tumors in rats fed the same peanut meal*
[From Newberne, Carlton, and Wogan, 1964, abridged. The peanut meals in groups 3 through 7 were contaminated with aflatoxins]

Group No.	Peanut meal		4 weeks wt. (gm)	Bile duct[1] hyperplasia in ducklings	Liver tumors in rats
	Type	Amt. in Diet (%)			
1	Expeller	5	(2)	0.0	-------
2	Expeller	20	(2)	4.0	-------
3	Expeller	50	585	10.0	4/10
4	Expeller	50	530	30.0	11/15
5	Expeller	50	569	30.0	7/10
6	Solvent	50	493	20.0	5/9
7	Solvent	50	525	16.0	8/15

[1] Graded 0 through 4+ based on degree of bile duct hyperplasia. These figures were multiplied by ten and averaged.
[2] Sacrificed at the end of two weeks.

Reduction of total body weight and liver weight along with bile duct hyperplasia parallel findings with contaminated peanut meal. Table 53 illustrates the close correlation between bile duct hyperplasia in ducklings and liver tumor induction in rats fed from the same lots of contaminated peanut meal.

Effects of feeding peanuts and wheat inoculated with a strain of *A. flavus* and of crude extract and purified fractions of extract on ducklings in seven days is shown in table 54. These results indicate that comparable lesions can be induced by 500 micrograms of crude liquid extract or (in micrograms) 7.8 crude wheat extract, 2.0, 15.6, or 50 of fractions B_1, G_1, and B_2 respectively. Such studies illustrate the relative potency of the various fractions of the aflatoxin complex in the duckling.

In acute studies with ducklings necropsy reveals a pale, atrophic liver (fig. 77) in which, on microscopic examination, is found widespread parenchymal cell necrosis and marked bile

TABLE 54.—*Effects of aflatoxin contaminated peanut meal and wheat and of crude and purified aflatoxin fractions on ducklings in 7 days*

Diet	Supplement	Initial weight (g)	7-day weight (g)	Liver weight		Average bile duct hyperplasia[1]
				Total (g)	Per cent body weight	
A (peanuts)	None	57	(2)	----------	----------	38.0
B (wheat)	None	55	144	5.5	4.7	30.0
C (control)	None	47	170	11.8	6.3	0
C	500 μg liquid culture extract	58	127	7.4	5.8	6.0
C	7.8 μg wheat culture extract	54	138	9.7	5.0	5.0
C	2.0 μg pure B_1	51	118	6.0	5.0	10.0
C	15.6 μg pure G_1	54	121	5.4	4.4	10.0
C	50.0 μg pure B_2	57	147	7.1	4.9	16.0

[1] Graded 0 to 4+ multiplied by 10 and averaged.
[2] Died before 7 days.

FIGURE 77.—The liver of the duckling on right is small and firm, typical of aflatoxin poisoning. Duckling on left is the control.

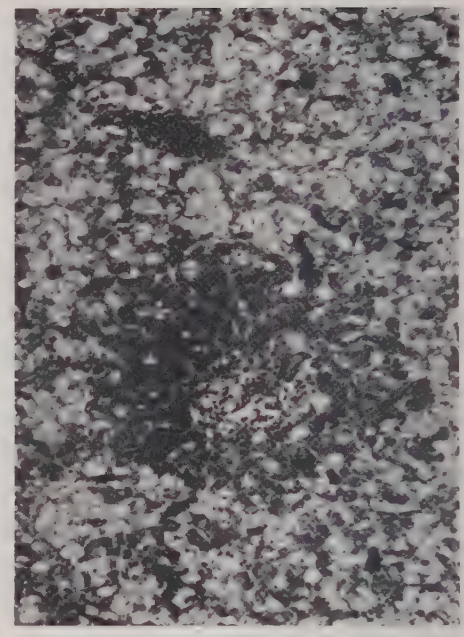


FIGURE 78.—Liver of duckling administered aflatoxin. Darkly stained proliferating bile ductule cells radiate out from periportal zone. H. & E. stain. × 210.

duct cell proliferation (figs. 78 and 79). The latter is induced very quickly and its degree is related to the amount and fraction of toxin consumed. This is the basis for the biological assay of aflatoxins in which the newly hatched duckling has proven quite valuable.

Subacute and chronic effects of aflatoxin in ducks is shown in table 55. In subacute cases of aflatoxicity in ducklings [*14*]—up to 30 days—there is nodular hyperplasia of parenchymal cells and massive bile duct hyperplasia (fig. 80). This can be induced by feeding peanut meal containing from 1.0 to 3.0 ppm of aflatoxin as 50 percent of the diet for 30 days. Long-term feeding studies—16 months—with ducks using the same diet resulted in severe cir-

TABLE 55.—*Subacute and chronic effects of aflatoxin-contaminated peanut meal on ducklings*

	Aflatoxin content (PPM)					
	<0.02 Control		1.0 Group I		3.5 Group II	
	30 Day[1]	16 Mo.	30 Day	16 Mo.	30 Day	16 Mo.
Avg. wt. gm	1450	3215	1020	2320	1300	2410
Liver						
Total, gm		83		44		46
% body wt.		2.6		1.9		1.9
Testes, total, gm		81		10		3
Bile duct hyperplasia	0/8	0/3	8/8	2/2	8/8	2/2
Nodular hyperplasia	0/8	0/3	3/8	2/2	5/8	2/2
Liver tumors	0/8	0/3	0/6	0/2	0/6	2/2

[1] The majority of ducks were taken off experiment for histopathologic study at the end of 30 days. A few were continued on experiment for a total of 16 months.

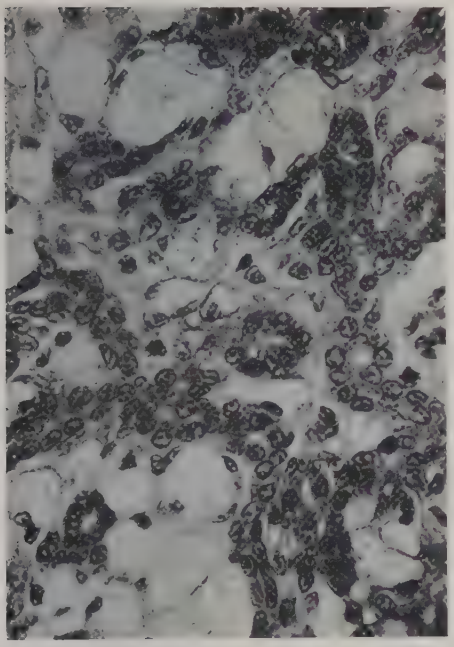


FIGURE 79.—High power view of proliferating bile duct cells some of which are forming duct-like structures. H. & E. × 475.

rhosis, bile duct hyperplasia and liver tumors (figs. 81–83). Effect on weight gains and testes development is also impressive.

The young chicken responds in a manner similar to the duckling but is much more resistant. The turkey and pheasant exhibit a response somewhere between that of the chicken and the duckling.

Outbreaks in chickens have resulted in moderate to severe losses in some cases, but generally mortality has been lower in chickens than in poults and ducks. Bile duct proliferation and hepatic cell degeneration is replaced after 2 weeks with massive lymphoid cell infiltration, provided the bird survives. Studies in our laboratory indicate that 5- or 6-week-old chickens require more than 4.0 mg/kg body weight to induce detectable effects.

Swine varying in age from 8 weeks to 1 year fed aflatoxin-contaminated peanut meal develop generalized jaundice. The liver is discolored, there is centrolobular necrosis, hemorrhage, and steatosis in acute cases, while in subacute or chronic cases there is perilobular fibrosis, pleomorphism of hepatic cells, and bile duct proliferation. Preliminary studies in our laboratory have shown that a dosage of 2.0 mg/kg body weight is an LD_{100} in weanling pigs.

Of the bovine species, calves are most susceptible to the effects of aflatoxin. Ascites and visceral edema are observed at necropsy. Atypical liver cells, bile duct proliferation, fibrosis, and veno-occlusive lesions are seen microscopically. The lesions are similar to those observed in seneciosis or ragwort poisoning.

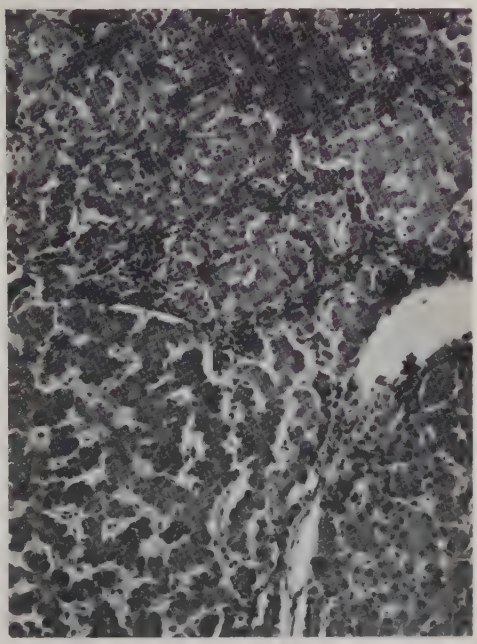


FIGURE 80.—Nodular hyperplasia and massive bile duct proliferation in duckling fed aflatoxin contaminated peanut meal for 30 day period. H. & E. × 120.

FIGURE 81.—Cirrhotic liver of duck fed aflatoxin-contaminated peanut meal for 16 months. Larger nodules are hepatomatous lesions.

Results of definitive studies on the effects of aflatoxin in guinea pigs have recently been reported by Butler and Barnes [*19*]. As pointed out earlier, evidence indicates that toxicity in guinea pigs has been observed since 1951 in sporadic outbreaks [*6*], but the etiology remained obscure until 1961 [*9*]. The spontaneous outbreaks have always been associated with feeding a pelleted diet which contained 15 percent groundnut meal. Paget [*6*] described the guinea pig syndrome as an exudative hepatitis characterized by gross subcutaneous edema, ascites, and marked liver changes. Butler [*19*] described the changes in the liver as bile duct hyperplasia with bizarre liver cells at the periphery of the lobule in the early stages. Fibrosis appeared as the disease progressed; this was accompanied by nodular hyperplasia of the

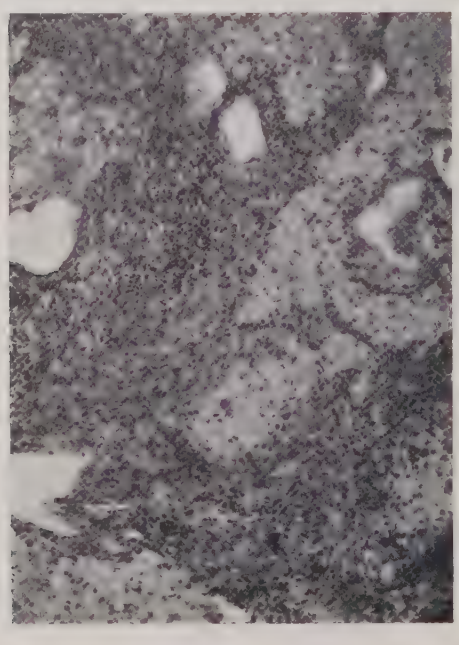


FIGURE 82.—Microscopic appearance of cirrhotic liver shown in figure 81. H. & E. × 100.

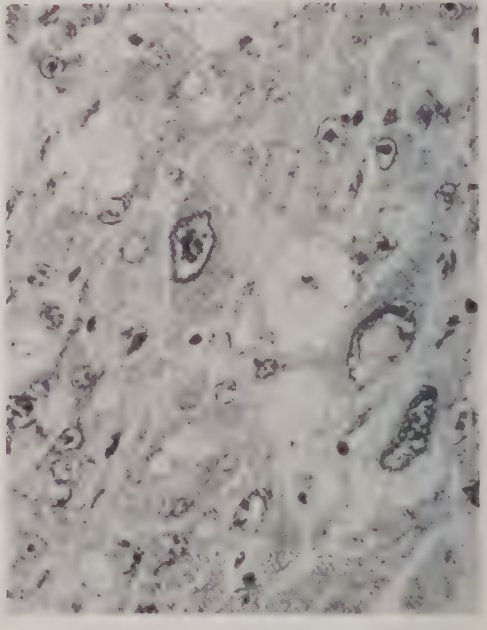


FIGURE 83.—Hepatomatous nodule of liver shown in figure 81. Note bizarre nuclei and vacuolated cytoplasm. H. & E. × 400.

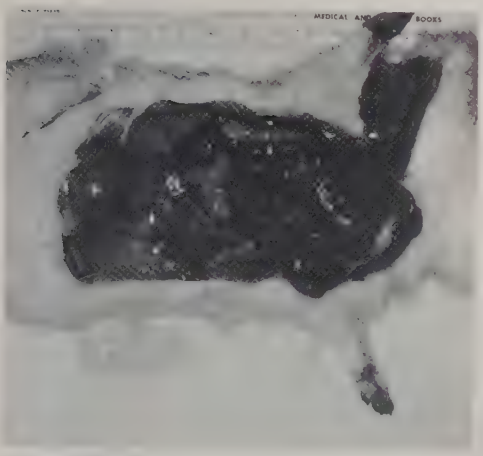


FIGURE 84.—Guinea pig administered aflatoxin dead in 48 hours with severe ascites, subcutaneous edema and swollen, fatty liver.

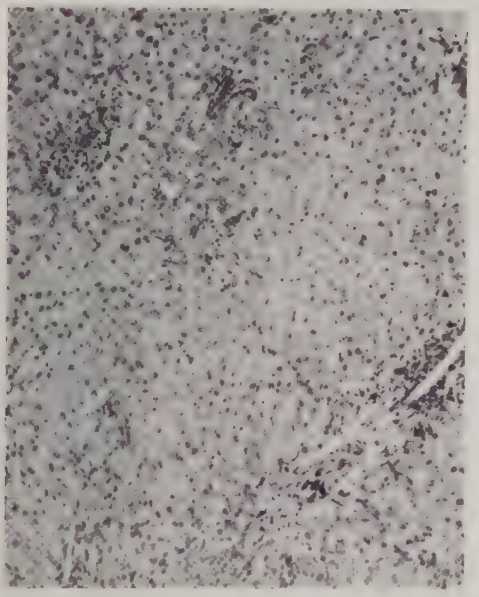


FIGURE 85.—Microscopic lesions observed in liver of guinea pig shown in figure 84. Note proliferating bile ducts about portal triad. H. & E. × 120.

parenchyma and marked thickening of the capsule. These lesions occurred in animals fed a diet with 0.35 to 0.4 ppm of aflatoxin where survival time was about 11 months. We have done acute studies (figs. 84 and 85) in the guinea pig and found essentially the same lesions as described by others. In weanling guinea pigs 0.5 mg/kg is an LD_{100} dose. We have not studied lower doses in this species.

Although the rat is comparatively resistant to acute toxic effects of aflatoxin, prolonged feeding results in liver tumors [*14,19,23*]. The tumors are malignant hepatocellular carcinomas, (figs. 86–88) many of which metastasize to the lung (fig. 89). The malignant tumors are accompanied by cystadenomas of bile ducts and by renal damage. Table 56 shows the response of rats to chronic feeding of aflatoxin-contaminated peanut meal, and it can

TABLE 56.—*Incidence of liver tumors and average body weight of rats fed peanut meal as major source of protein**

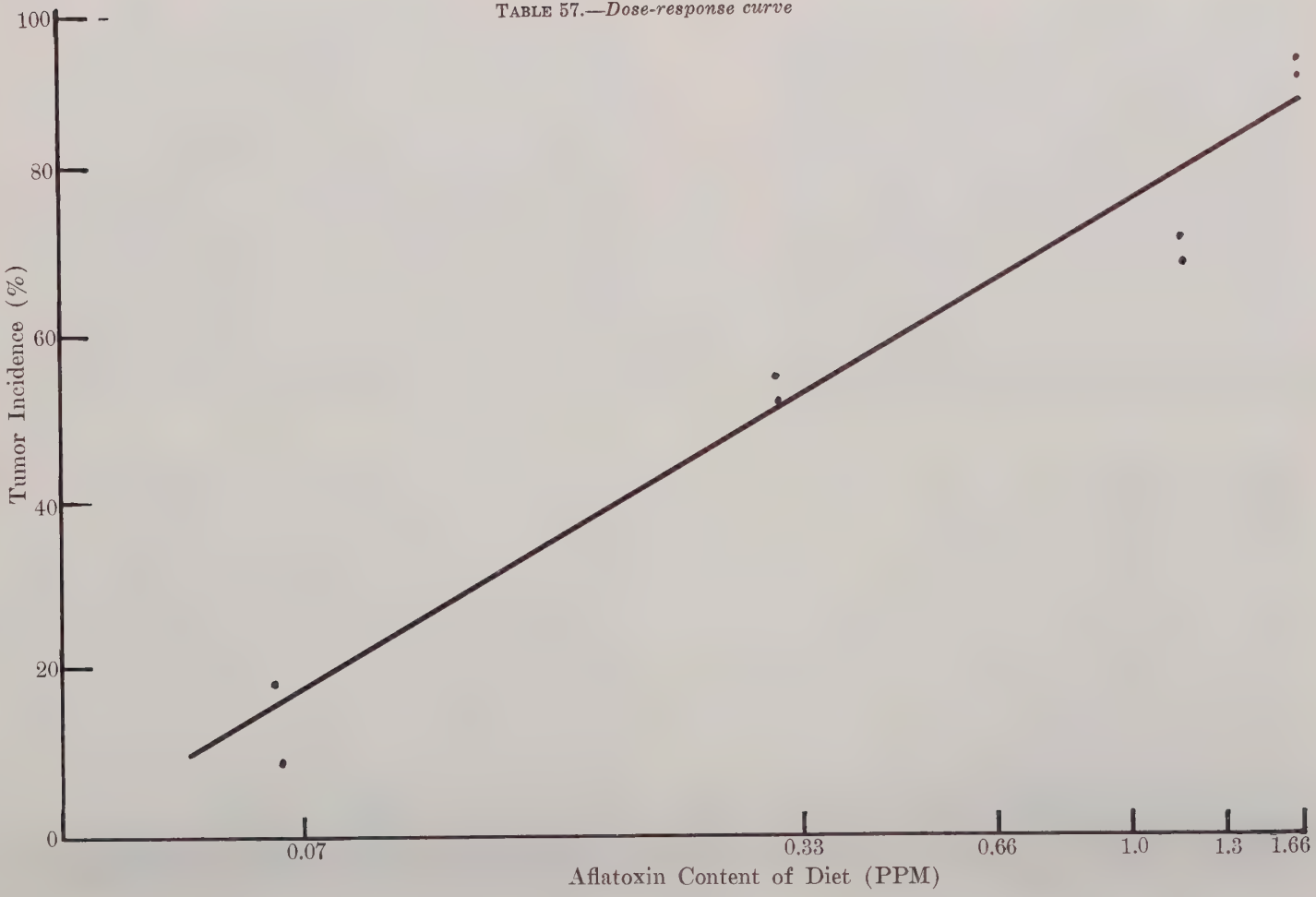
Group No.	Peanut meal treatment		Petroleum solvent + MeOH ext.	Aflatoxin** content of diet ppm	Max. avg. wt. (gm)	Avg. duration (days)	Tumor incidence
	Expeller	Petroleum solvent ext.					
1	+	−	−	1.70	763	370	14/15
2	+	−	−	1.17	719	340	11/15
3	+	−	−	1.17	640	335	7/10
4	−	+	−	0.33	793	294	5/9
5	−	+	−	0.33	738	323	8/15
6	−	−	+	0.07	715	360	2/10
7	−	−	+	0.07	714	361	1/10
8	+	−	−	0.007	786	384	0/10

* Expeller peanut meal has been exposed to hydraulic pressing only which removes all but about 8% of oil; petroleum solvent meal is expeller meal further treated with petroleum solvents, principally n-hexane to reduce fat content to 0.5–1.0%. This meal is further extracted with hot methanol which removes all detectible fat. From Newberne, Carlton, and Wogan, 1964, abridged.

** Aflatoxin content determined by fluorescence assay.

222-175 O - 67 - 10

TABLE 57.—*Dose-response curve*



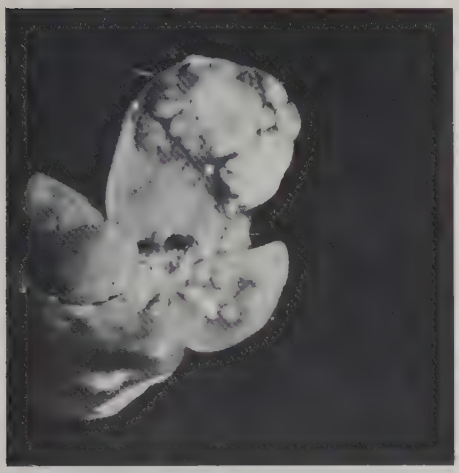


FIGURE 86.—Rat liver tumor associated with aflatoxin. Large nodule is malignant and metastatic.

be observed that there is a well-defined dose-response relationship in this species (table 57). Calculations indicate that a total dose of between 2 and 3 milligrams will induce liver tumors.

Mice have shown a resistance to aflatoxin in our laboratory similar to that exhibited by sheep except that one strain of the former species developed a low incidence of liver tumors after prolonged feeding (16 months) of aflatoxin-contaminated peanut meal (1 ppm). The tumors (fig. 90) arose from an otherwise smooth liver lobule and reached a size of 2 to 3 centimeters in diameter.

Recent work in our laboratory with acute studies in dogs indicates that the earlier reported outbreaks in dog kennels of Hepatitis X were probably in part a result of aflatoxin contamination of commercial feed. Experimental lesions are morphologically identical to those observed in field outbreaks except

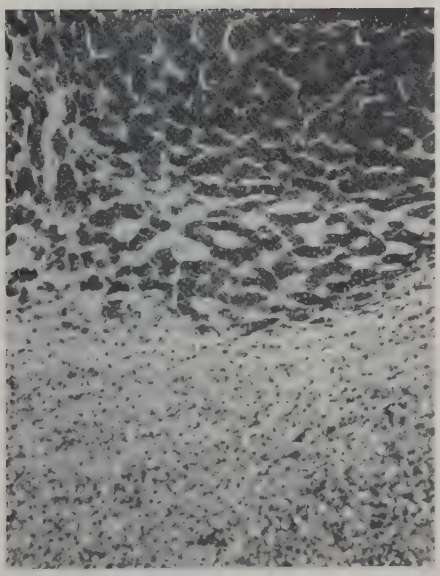


FIGURE 87.—Microscopic appearance of liver tumor. Rapidly proliferating parenchymal-type cells are replacing more normal liver tissue. H. & E. × 100.

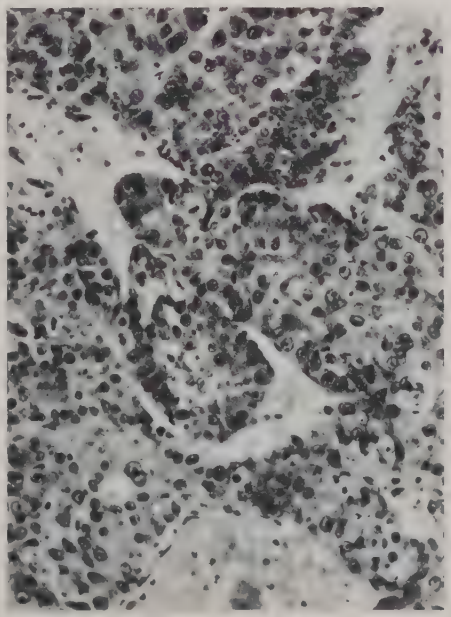


FIGURE 88.—Trabecular, vascular component of tumor shown in figure 86. H. & E. × 225.

that nodular regeneration of the parenchymal cells is much less pronounced. In this species, 0.5 mg/kg body weight, orally or intraperitoneally, is an LD_{50} dose. It results in severe ascites (fig. 91) fatty infiltration of the liver (figs. 92 and 93), severe gall bladder edema (fig. 94), bile duct hyperplasia (fig. 95), and occasionally, hemorrhage into the gastrointestinal tract.

Studies with rabbits in our laboratory have shown them to be quite sensitive to the effects of aflatoxin with an LD_{50} of about 0.3 mg/kg body weight. Cats have shown a similar sensitivity. Lesions in both rabbits (fig. 96) and cats (fig. 97) are similar to those described in other species: liver parenchymal degeneration, bile duct hyperplasia and occasional hemorrhage.

Our attempts over the past several years to induce choline-deficiency liver tumors in the rat have been totally unsuccessful. Sources of dietary pro-

FIGURE 90.—Liver tumor of mouse fed aflatoxin-contaminated peanut meal for 16 months. Large nodule arose from right liver lobe while remainder of liver was grossly normal.

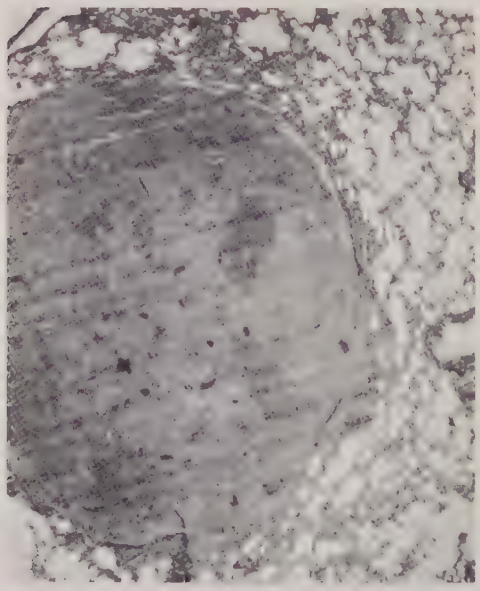


FIGURE 89.—Metastatic lesion of tumor in lung. Tumor emboli were observed also in pulmonary vessels. H. & E. × 60.

FIGURE 91.—Dog, severe ascites and petechiae seen through intestinal wall lower right, as a result of aflatoxin.

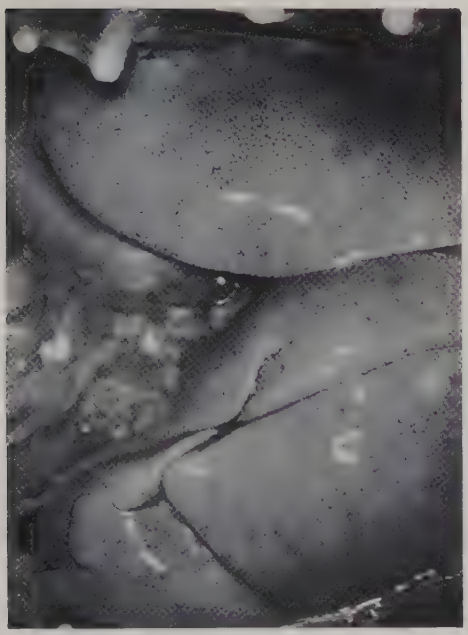


FIGURE 92.—Greatly enlarged and fatty liver in puppy administered aflatoxin. Note also mesenteric edema.

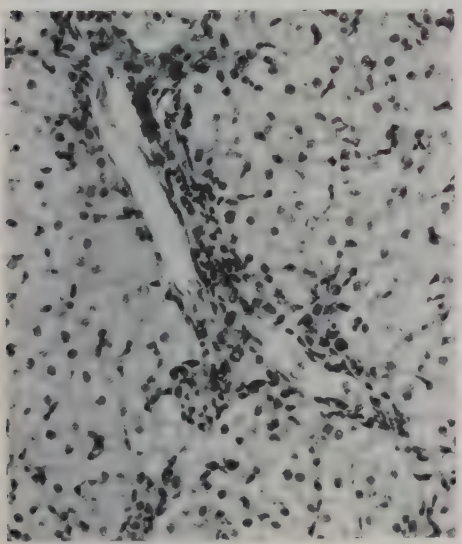


FIGURE 93.—Microscopic appearance of liver shown in figure 92. All parenchymal cells are distended with lipid which appears as vacuolated space about dark staining nucleus. H. & E. × 400.

tein have included vitamin-free casein (commercial), casein extracted with methanol in our laboratory, water-washed alpha-protein, methanol extracted peanut meal and combinations of these. In feeding experiments involving several hundred rats for periods up to 19 months we have succeeded only in inducing liver cirrhosis such as that shown in figures 98 and 99. Liver tumors have been observed only in rats fed peanut meal as a source of part of the protein; in most cases chemical and biologic assay have shown that the peanut meal was contaminated with aflatoxin. An interesting sidelight has been the observation that small doses of aflatoxin administered daily for 2 to 3 weeks to rats fed cirrhogenic diets resulted in liver cirrhosis in a total time of only six weeks (figs. 100–102). This

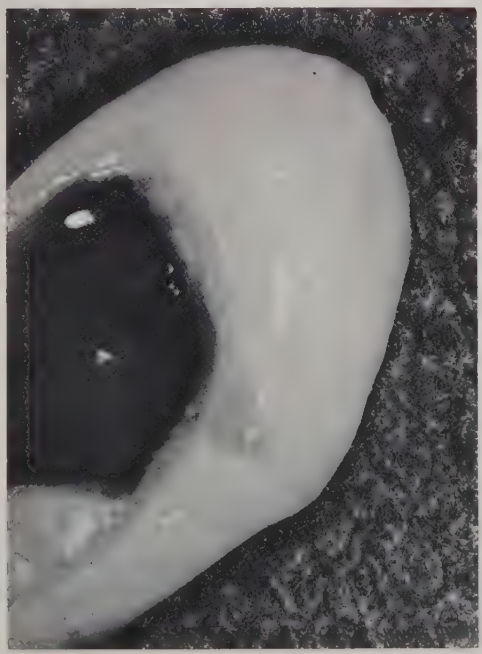


FIGURE 94.—Gall bladder from liver shown in figure 92. Characteristic lesions are shown including severe subserous edema and edema with hemorrhage in mucosa.

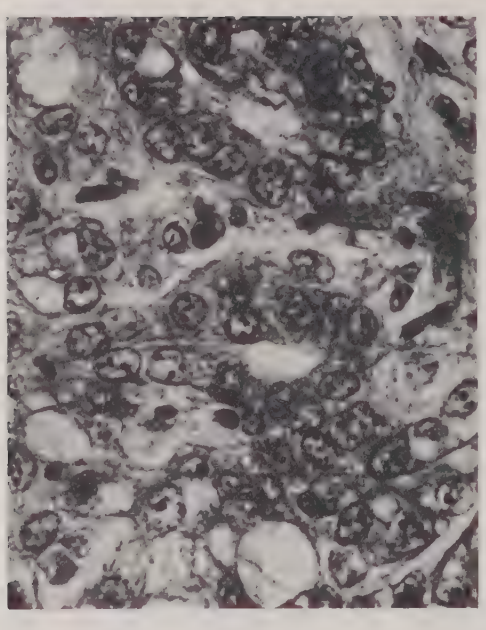


FIGURE 95.—Bile duct hyperplasia, characteristic of aflatoxicity, observed in liver of dog given aflatoxin by capsule at rate of 0.5 mg/kg body weight sacrificed 14 days later. H. & E. × 1000.

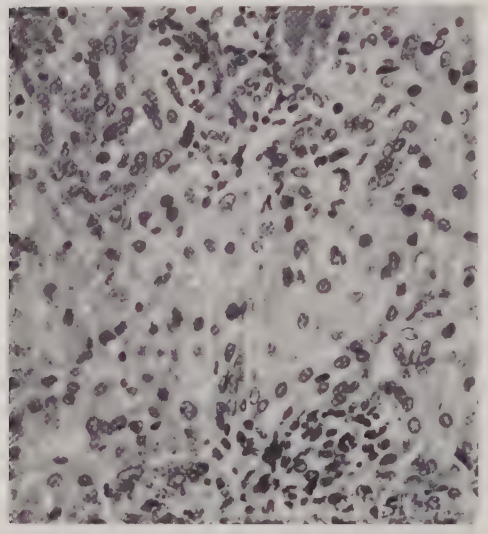


FIGURE 96.—Bile duct hyperplasia observed in rabbit given aflatoxin as a single dose at rate of 0.3 mg/kg body weight sacrificed 5 days later. Many rabbits succumbed in 24–36 hours with this dosage. H. & E. × 400.

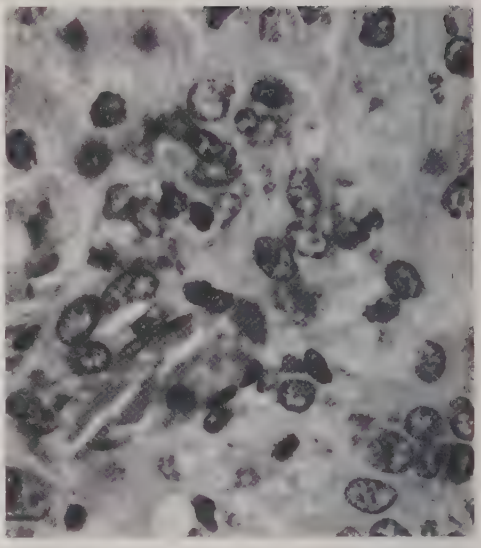


FIGURE 97.—Section from liver of cat sacrificed 72 hours after 0.5 mg/kg body weight as a single dose of aflatoxin administered intraperitoneally. H. & E. × 1000.

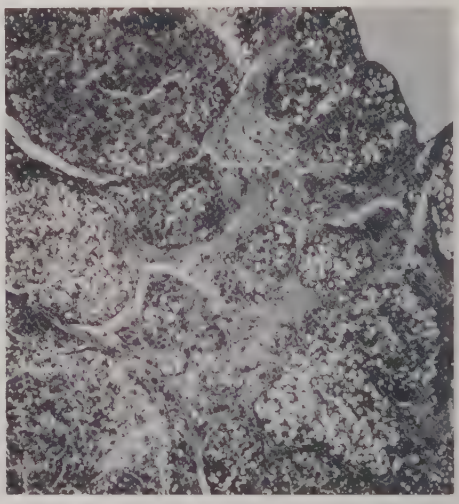


FIGURE 98.—Liver cirrhosis induced in rat, typical of that observed in animals fed cirrhogenic diets containing casein, alpha-protein, peanut meal and combinations of these for periods up to 19 months. The nodules are hyperplastic parenchyma only; liver tumors were observed only in rats fed diets contaminated with aflatoxin. H. & E. × 55.

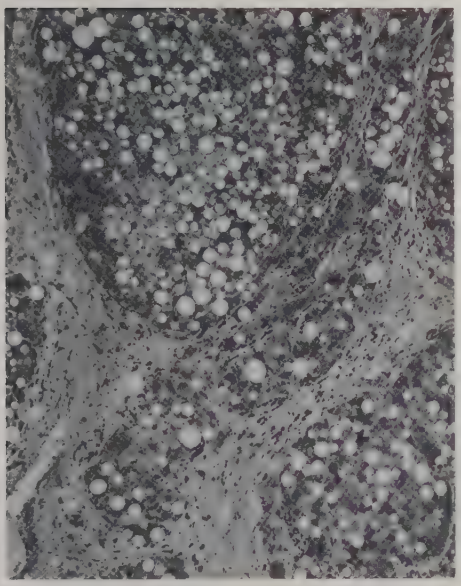


FIGURE 99.—Fatty, nodular hyperplasia in cirrhotic liver with interlacing bands of connective tissue and scattered proliferating bile ducts. H. & E. × 100.

FIGURE 101.—Low power microscopic appearance of liver shown in figure 100. Nodular regeneration and ductule cell hyperplasia is marked. H. & E. × 40.

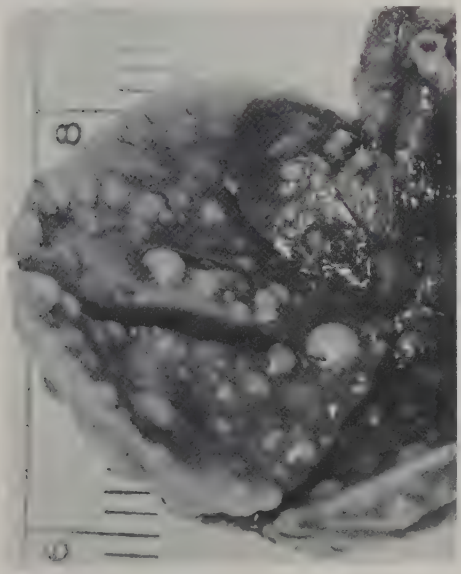


FIGURE 100.—Cirrhotic liver from rat fed cirrhogenic diet for 6 weeks from time of weaning. Low levels of aflatoxin were administered daily for first 2 weeks by intubation.

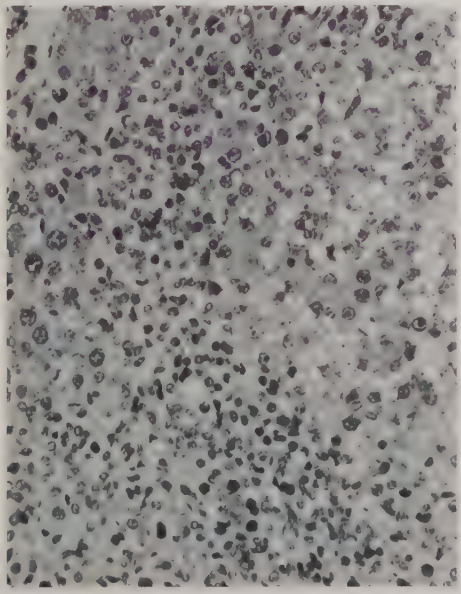


FIGURE 102.—High power microscopic appearance illustrating fatty infiltration into proliferating parenchyma and broad band of diffuse bile ductule cells. H. & E. × 400.

is a much shorter period than that usually required to induce cirrhosis by the cirrhogenic diet alone.

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AFLATOXINS IN FEEDS—A REVIEW

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Abstract.—Chemistry, biological effects, occurrence and measurement of a class of mold toxins known as aflatoxins have been reviewed. Aflatoxins have a known structure, affect many species of animals, exert their primary effect on the liver in a similar but not identical manner, are extremely toxic and carcinogenic, have appeared in most areas of the world, are easily removed from vegetable oils but continue to be a problem in feedstuffs, such as peanut meal. Prevention of aflatoxin contamination by proper control of moisture and temperature, especially in the rapid drying of peanuts after removal from the ground, should be urged. Research is continuing to develop methods of removal from contaminated products. The vast majority of food grade peanuts are free of aflatoxins, and it appears to be only a matter of time until all feeds can be made free of these recently discovered compounds.

Although molds in feeds have been a problem for a great many years, events occurring during only the past four years have given a tremendous impetus to mold toxin research. Deaths of thousands of poultry and poor response in other animals in England and elsewhere have been attributed to moldy peanut meal. On one farm alone 14,000 ducklings died after ingesting a ration containing imported Brazilian peanut meal, and it has been estimated that 100,000 turkeys died in England in 1960 from the then mysterious disease dubbed "Turkey X" disease. Eventually the cause of the disasters was traced to a series of metabolites of *A. flavus* which initially were collectively called aflatoxin. Because of their toxicity and carcinogenicity the aflatoxins continue to receive considerable attention from various private and governmental groups.

Much has been written about the disasters in England attributed to moldy peanut meal. For details and reviews of this and other mycotoxicoses the following references may be found helpful: *3, 6, 7, 8, 9, 15, 16, 37, 42, 62, 74,* and *79.*

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CHEMISTRY OF AFLATOXIN

Although this paper is devoted mainly to aflatoxins elaborated by *Aspergillus flavus* and *Penicillium puberulum* Bainer [*47*], additional toxins are also elaborated by *A. flavus.* The reader interested in the latter toxins should consult the papers by Tilden et al. [*81*] and Wilson and Wilson [*86*].

It has been reported that aflatoxin production takes place in the mold mycelium and most of the toxins are secreted in the medium with trace amounts in the spores [*18*].

As mentioned previously aflatoxin is a collective term. There are at least four types of aflatoxins, designated B_1, B_2, G_1, and G_2, the letters having come from whether they fluoresced blue or green under ultraviolet light. Some researchers have used other letter designations and variations in fluorescence have been noted with intensity of fluorescence being inversely proportional to

degree of induced toxicity [*24*]. For further details the following references should be consulted: *8*, *13*, *24*, *29*, *32*, *33*, *45*, *56*, *78*, and *84*. Although as many as twelve ultraviolet fluorescent compounds appear to be elaborated by *A. flavus*, of which five have been reported to be toxic [*78*], it is quite possible that isolation procedures and exposure to light result in the formation of derivatives of the natural toxins. At any rate, the structures for aflatoxins B_1, B_2, G_1, and G_2, based upon the work of scientists from several countries [*13*, *25*, *26*, *45*, *83*, and *85*], appear to be defined.

It is interesting to note the coumarin nucleus since a previous mold problem, sweet clover poisoning, was related to altered coumarin in the plant [*6*]. Furthermore, biosynthesis of aflatoxins from aromatic amino acids resembles the proposed synthesis route of coumarin [*1*].

The aflatoxins are chloroform and methanol soluble but insoluble in petroleum ether [*13*, *56*], melt with decomposition in the range from 230° to 309° C [*13*, *25*, *45*, *83*, *85*] and have been reported to occur in the ratios of 40 B_1:1 B_2:50 G_1:1 G_2 [*53*], 15 B_1:1 B_2:15 G_1:1 G_2 on a weight basis [*47*], and, on a molar basis, 118 B_1:6 B_2:11 G_1:1 G_2 [*1*]. Only the B_1 and B_2 components appear to be elaborated by some strains of *A. flavus* indicating that the green aflatoxins may be oxidized forms of the blue components and that certain strains of aspergilli lack the necessary enzymes to carry out the oxidation [*29*].

FUNGAL SPECIES PRODUCING AFLATOXINS

At least a dozen strains of aflatoxin-producing aspergilli and one penicillium are known, but the amount and types of aflatoxin produced appear to be variable [*1*, *16*, *29*, *45*, *47*, *79*]. Although aflatoxins display ultraviolet fluorescence some fluorescent isolates of *A. flavus* are nontoxic [*16*, *79*]. Diener et al. [*36*] found no indication of toxin production when *A. amstelodami*, *A. chevalieri*, *A. ruber*, *A. terreus*, *A. restrictus*, *P. citrinum*, *A. candidus*, and *A. micro-virido* were cultured on autoclaved peanuts and the inoculated peanuts defatted, ground and fed to ducklings. The feeding period, however, was limited to three days. On the other hand, Codner et al. [*29*] found that *A. parasiticus* produced 265 mg of aflatoxins per kilogram of peanuts when cultured in the laboratory whereas much lower amounts were produced by different strains of *A. flavus*, ranging from 14 to 162 mg/kg of peanuts. All batches of aflatoxin, however, were found to be toxic to ducklings.

EFFECT OF ENVIRONMENT ON AFLATOXIN PRODUCTION

Relatively little appears to have been published in regard to the actual field conditions required for aflatoxin production although much is known about requirements for growth of *A. flavus*. In a review of molds on peanuts Austwick and Ayerst [*16*] suggested that invasion of peanuts with *A. flavus* occurred after the peanuts were removed from the ground and before the moisture content of the nuts was reduced to a safe level. Peanuts found to be toxic to ducklings could be separated from nontoxic peanuts solely by their appearance. The authors reported that less than 3 percent of the discolored, toxin-containing peanuts could be sufficient to render a sample toxic.

A recent series of papers from London and Nigeria has provided much useful information on field development of aflatoxins in peanuts [*18*, *53*, *54*, *55*]. The results showed: (a) *A. flavus* grew much more successfully on

moribund or dead peanut tissue than it did on living tissue. (b) There was virtually no toxicity in the crop at harvest except in broken pods. (c) Contamination of kernels with *A. flavus* and appearance of the toxin did not occur until at least five days after harvest. (d) Kernels from broken pods were more likely to be contaminated with *A. flavus* and to be toxic than were kernels from undamaged pods. (e) Toxicity of the crop varied from year to year at specific localities.

Several groups of workers have shown that aflatoxins can be produced by culturing *A. flavus* on synthetic media as well as on natural feedstuffs [*1, 12, 32, 47, 56, 61*].

TOXICITY OF AFLATOXINS

Acute toxicity

The extreme toxicity of aflatoxins is demonstrated by the fact that the extract of less than 2 grams of toxic peanut meal has been shown to kill a day old duckling [*74*]. LD_{50} values with day old ducklings for aflatoxin B_1 have been reported to be 30–50 μg (0.5–0.9 mg/kg) [*84*], 30 μg (0.5 mg/kg) [*53*], 28.2 μg (0.5 mg/kg) [*13*], and 18 μg (.5 mg/kg) [*24*]. LD_{50} values for aflatoxin G_1 have been reported as 60 μg (1.1 mg/kg) [*45*], 90 μg (1.8 mg/kg) [*13*], and 39 μg (1.0 mg/kg) [*24*]. Aflatoxins B_2 and G_2, reduction products of B_1 and G_1, have reported LD_{50} values with day old ducklings of 85 μg (2.2 mg/kg) and 172 μg (4.5 mg/kg) [*24a*]. Although aflatoxins B_2 and G_2 are far less toxic than the respective B_1 and G_1, they do induce liver lesions in ducklings characteristic of the lesions induced by aflatoxins B_1 and G_1 [*24, 25, 45*]. Evidence has been presented to indicate that when either aflatoxins B_1 or G_1 are administered to ducklings with the corresponding reduction product, B_2 or G_2, acute toxicity is enhanced by presence of the reduction product [*32*]. Also addition to the ration of basic amino acids appeared to enhance toxicity attributed to aflatoxins [*43*] despite the fact that addition of lysine has been shown to counteract growth depression of experimentally molded soybean meal [*67*].

Studies with rainbow trout have established a tentative LD_{50} value of 1.5 mg/kg for a mixture of aflatoxins B_1 and G_1 [*14*]. A similar LD_{50} value of 1.4 mg/kg for ducklings given a mixture of aflatoxins has been reported [*11*]. A mixture of aflatoxins produced by *P. puberulum* Bainer has shown an LD_{50} value of 2.3 mg/kg when administered to ducklings [*47*].

Chronic toxicity

An excellent review of aflatoxin and toxic peanut meal effects in animals appears in the paper by Allcroft and Carnaghan [*3*]. Among poultry, ducklings appear to be the species most sensitive to aflatoxins, with turkeys being less sensitive and chicks relatively resistant. Among larger animals, pigs are more sensitive than young cattle, older cattle are less affected and sheep are comparatively resistant. Ducklings fed feces of sheep which received aflatoxins did, however, display aflatoxicosis [*2*]. Mice have shown no symptoms of injury after consuming as much as 80 percent ration level of moldy peanut meal containing more than 5 ppm of aflatoxins [*63*]. Young of a species are more susceptible to aflatoxins than are older animals which has prompted a recommendation that toxic peanut meal be excluded from rations of calves less than 3 months of age [*9*]. Comparative species effects for chicks and poults were noted by Gardiner [*40*] and results among several species of domestic animals fed known levels of aflatoxins [*9*] are summarized

in table 1. Additional comparative effects of aflatoxins between rats and ducklings were reported by Newberne et al. [*59*].

Clinical symptoms reported for aflatoxin and/or moldy peanut meal toxicity include inappetance and reduced growth rate for monkeys [*82*], swine [*9, 52, 56*] rats [*48*], cattle and calves [*5, 9, 51, 52*], ducklings [*15, 23, 25, 61*], chickens [*15*], and turkeys [*3*]. Other prominent symptoms include lameness in ducklings [*15*], reduced milk yield for cows [*5, 52*], and, in beef cattle, rough hair coats, amaurosis, aimless walking in circles, frequent falling, ear twitching, grinding of teeth and eversion of rectum [*5, 51*]. Some workers have reported that toxic peanut meal does not reduce growth rate of rats [*59*].

HISTOLOGICAL EFFECTS OF AFLATOXINS

The most prominent pathological effect produced by ingestion of aflatoxins or *A. flavus*-contaminated feedstuffs occurs in the liver of animals. Bile duct proliferation or hyperlasia and/or liver parenchymal cell degeneration has been reported for ducklings [*11, 12, 15, 23, 25, 47, 58, 59, 61, 72, 73, 87*], turkeys [*3, 72*], chickens [*15*], pheasants [*23*], cattle [*5, 28, 51*], swine [*44, 50*], rats [*22, 48, 71, 75*], guinea pigs [*22*], and monkeys [*82*]. Liver fibrosis, necrosis and enlarged liver cell nuclei are also prominent features of toxicity associated with ingestion of *A. flavus*-contaminated peanut meal or aflatoxins [*3, 5, 12, 15, 22, 28, 35, 36, 44, 50, 51, 61, 72, 82*]. Effects on kidney [*15, 22, 28, 36, 44, 48, 59*], pancreas [*15, 36*], lung [*22, 48*], stomach [*22*], and salivary glands [*22*] have also been noted.

Liver and other tumors have been produced in rats by feeding peanut meals, presumed to have been contaminated with fungal toxins [*48, 49, 59, 60, 68, 71, 75*]. In some studies the level of aflatoxin in the meal was measured and found to occur up to 8 ppm of B_1 [*22*]. Results from feeding high levels of domestic peanut meals are shown in table 59. Additional information is given in a later publication [*59*].

Although aflatoxins were presumed to be the cause of tumor formation, proof was lacking until Dickens and Jones [*35*] established that subcutaneously injected, relatively pure aflatoxins induced cancer in rats. Additional studies published by Barnes and Butler [*19*] showed that rats fed no more than 2.5 mg of aflatoxins of 80

TABLE 58.—*Effect on domestic animals of graded levels of aflatoxins*
[Reference *9*]

Species	Type of feeding	Ration level of aflatoxins, ppm	Results		Liver lesions
			+ Reduction of		
			Growth	Feed effic.	
Swine	Restricted	0.14	0	0	0
Do	do	0.28	+ (10%)*	+ (6%)*	0
Do	do	0.41	+ (13%)*	+ (8%)*	+ (minimal)
Do	Ad lib.	0.41	+*	+*	+ (minimal)
Do	do	0.69	+*	+*	+ (yellow)
Do	Restricted	0.69	+*	+*	+
Cattle (2 yrs. old)		0.22	0	0	0
Do		0.44	0	0	0
Do		0.66	0	0	+
Calves, dairy (1–3 mos.)		0.22	+	+	+
Do (3–4 mos.)		0.22	0	0	0
Do (1–3 mos.)		0.44	+	+	+
Do (3–4 mos.)		0.44	0	0	0

* Effects noted only when pigs in growing stage 40–140 lb; no effect in finishing stage (140–200 lb).

percent purity developed liver cancer. Similarly a high incidence of hepatomas in rainbow trout has been induced by feeding aflatoxins [*14*]. On the other hand, no tumors have been observed in pigs, calves, and cattle fed up to 0.69 ppm ration level of aflatoxins [*9*].

TABLE 59.—*Liver tumors in rats fed domestic peanut meals containing aflatoxins*

[Newberne et al., *60;* male rats received peanut meal as 75% of dietary protein for 9–12 months]

Peanut meal	Meal content of aflatoxins, ppm	Rat hepatomas	
		Number	%
Solvent C	.005	0/10	0
Expeller A	.03–0.5	1/7	14
Solvent D	1.0	4/9	44
Expeller B	3.0–3.5	5/5	100

BIOCHEMICAL EFFECTS AND MODE OF ACTION OF AFLATOXINS

Biochemical changes induced by toxic peanut meal or *A. flavus* toxins include increased serum alkaline phosphatase, sometimes followed by a decrease, in monkeys [*82*], swine [*44*], and calves [*5*], and decreased liver vitamin A in swine [*44*] and calves [*5*]. Decreased vitamin A levels in animals fed corn inoculated with nontoxic samples of *A. flavus* have also been reported [*21*]. Glutamate-oxalacetate transaminase (G.O.T.) activity in serum of calves fed toxic peanut meal was within the "normal range" [*5*], but increased G.O.T. activity, indicative of cell destruction, was noted in serum of swine fed toxic peanut meal [*44*] and in serum and liver of monkeys fed purified aflatoxins [*82*].

The mode of action of aflatoxin needs further elucidation but Lancaster et al. [*48*] reasoned that presence of rat liver tumors in the absence of cirrhosis, cell necrosis, or cellular infiltration suggested that the aflatoxins act directly on the cell. Smith [*77*] showed that purified aflatoxins reduced the incorporation of C^{14} labeled leucine into protein of rat and duckling liver slices. Aflatoxins did not appear to inhibit incorporation of labeled leucine into rat kidney slices but distinct vacuolation of monkey kidney cells with subsequent growth inhibition and cell destruction was induced by aflatoxins.

In contrast to the report of Smith [*77*], Shank and Wogan [*76*] found that incorporation of C^{14} from labeled glucose and leucine was reduced in liver lipid and increased in liver protein of animals fed aflatoxins. *In vivo* studies by the same authors also showed that aflatoxin B_1 feeding resulted in decreased liver glycogen and increased liver lipid of ducklings and rats. A similar increased liver fat and decreased protein in liver and serum was reported for monkeys fed a mixture of aflatoxins B_1 and G_1 [*82*]. Other changes noted in monkey serum were decreased albumin and albumin/globulin ratio and increased serum bilirubin.

ASSAY OF AFLATOXINS

Both chemical and biological methods for assaying aflatoxins are available and are being continually revised and improved.

Chemical

Thin layer chromatographic (TLC) [*1, 10, 20, 32, 33, 34, 57, 66*] and paper chromatographic [*30*] chemical methods have been developed for measurement of aflatoxins in peanuts and other foodstuffs. Aflatoxins B_1, B_2, G_1, and G_2 have been detected by the methods of Adye and Mateles [*1*] and de Iongh et al. [*32*]. Coomes et al. [*30*] reported that the paper chromatographic method detects as little as 0.1 μg of pure aflatoxin B_1 whereas the much more sensitive TLC methods detect as little as 0.003 μg of aflatoxin B_1. The authors also cautioned that amount of aflatoxins may be overestimated in some samples,

such as expeller produced oilseed meals.

Oscillographic polarography has been used to detect relatively pure samples of aflatoxins B_1 and G_1 but the technique does not appear to be useful for routine analysis [*39*].

Caution should be exerted during analysis of aflatoxins not only because of their extreme toxicity and carcinogenicity but because exposure to light and alkali can have a profound effect upon quantitative results [*84*].

Biological

Biological assay of aflatoxins based upon liver lesions in ducklings has been reported and appears to be very useful for detection of aflatoxin contaminated materials [*3, 11, 60, 61, 72, 74, 87*]. Duckling liver response depended upon dosage schedule [*11, 87*] and microscopic liver changes were not observed until multiple dosage level of mixed aflatoxins given to 55 g ducklings exceeded 10 μg/kg [*11*] or, for aflatoxin B_1, 2 μg [*61*] or 7 μg/kg [*25*]. With single dosage a level of 100 μg/kg of mixed aflatoxins was required to induce liver lesions [*11*]. Aflatoxin G_1 and B_2 induced liver lesions in ducklings where administered at levels of 16 and 50 μg respectively [*61*].

Preliminary results of a chick embryo bioassay for aflatoxins, reported to be more sensitive than the duckling test, have been published [*64*].

AFLATOXINS IN FEEDSTUFFS OTHER THAN PEANUT MEAL

Although peanut products received the bulk of the publicity it seemed unlikely that occurrence of aflatoxins in feedstuffs would be limited to peanuts. Strong evidence has been reported for production of aflatoxin by toxic strains of *A. flavus* or *P. puberulum* cultured on sterilized runner beans [*69, 70*], soybeans [*12, 69*], cottonseed [*69*], wheat [*12, 25, 47, 61, 69*], rye [*12*], buckwheat [*12*], corn or maize meal [*12, 18, 21, 83*], rice [*12*], potatoes [*41a*], and peanut meal-free poultry feed [*69*]. In addition, natural feedstuffs other than peanuts or peanut meal, such as tropical "maize meal" [*3*] and Sudanese cottonseed meal [*52*], have been reported to be contaminated with aflatoxins. One writer, however, in referring to aflatoxins concluded, "—and none seems to be produced when the mold grows on cottonseed meal." [*65*]. Very little of the tremorgenic *A. flavus* toxin is produced when *A. flavus* is grown on peanut meal or timothy hay, but a considerable amount appears when the substrate is oats, millet, rice, potatoes, or corn [*86*]. Since this toxin appears to be inactivated by relatively mild heating [*81*], in contrast to the aflatoxins which withstand temperatures in excess of 150° C. for several hours [*16*], it may prove to be much less of a problem than the aflatoxins.

The possibility of feedstuff contamination within the poultry house becomes apparent when one considers that *A. flavus* constituted more than half of the genera of fungal flora found in hatcheries [*27*] and toxin-producing strains of *A. flavus* have been found in poultry houses [*69*].

AFLATOXINS IN FOODS

Under experimental conditions, aflatoxins appeared to have been secreted in milk of rats fed pure aflatoxin B_1 [*33*] and of cows fed toxic peanut meal [*4, 33, 38*]. Slight modification of the toxin occurred [*33*] and the amount of toxin secreted in the milk was less than one per cent of the total amount of aflatoxin given to the cows during the experimental period [*38*]. The toxin appeared in the milk 12 to 24 hours after feeding toxin-containing peanut

meal and disappeared from the milk within four days after cessation of feeding. Bile duct proliferation characteristic of liver changes induced by aflatoxins appeared in ducklings fed the toxin-containing milk [*4, 33, 38*], but bulked samples of commercial milk were reported to be free of toxins [*4*].

When toxins were detected in the milk from cows fed toxic peanut meal the toxin appeared in the rennet-precipitated casein fraction of the milk proteins with no detectable toxins in protein-free filtrate [*4*]. The same report also showed that pig liver, cow blood, and hens' eggs from the respective animals fed toxic peanut meal did not appear to harbor aflatoxins. Although the British have reported some samples of peanut products intended for human consumption were toxic, they commented further, "In the course of many hundreds of tests we have not detected toxicity in any other foodstuff although this does not preclude the possibility that this may occur under unfavorable conditions of storage." [*3*]. In other reports British workers showed that alkali refining of peanut oils accounted for the absence of aflatoxins in peanut oil [*8, 46, 79*] and rigid selection of nuts accounted for absence of toxins in peanut butter [*8*]. Representatives of the Canadian Food and Drug Directorate reported, "Eleven peanut butter samples purchased in Canada and probably made from peanuts grown in the U.S.A. were found to contain no toxic material." [*41*]

CONTROL OF THE AFLATOXIN PROBLEM

Obviously, prevention of aflatoxin production and contamination is preferable to counteraction of the toxins in feeds or foods. Although literature on moisture and temperature requirements for molds is voluminous; data relating directly to aflatoxin production is meager. Austwick and Ayerst [*16*] have, however, contributed helpful information in their review which noted that *A. flavus* would grow at or above 75 percent relative humidity in a temperature range of 30° to 38° C. (86°–100° F.). The series of papers from London and Nigeria [*18, 53, 54, 55*] showed that aflatoxins in peanuts usually develop after harvest and prior to drying. Thus it appears prudent to rapidly reduce feedstuffs to a moisture level where molds will not grow and to maintain this low moisture level by dry storage conditions. Since mold growth is also temperature dependent, maintenance of a low storage temperature is desirable.

No attempt will be made to assess the feasibility of adding mold inhibitors to feedstuffs. Such materials have, however, been used to control molds in feedstuffs as well as foodstuffs.

As mentioned previously, aflatoxins appear to be removed from vegetable oils by alkali refining; but conventional solvent (commercial hexane) extraction or pressing does not remove aflatoxins from oilseed meals. New processing methods, among them extraction with more polar solvents, are being investigated as a practical means of removing aflatoxins from feedstuffs, but it has been reported that ether and ethanol will not remove the toxins [*80*]. The extreme heat stability of the aflatoxins greatly complicates the problem of inactivation.

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Discussion

DR. PHELPS: Mold toxins, and more specifically, aflatoxins, appear to be present in many agricultural commodities. Although many types of mold toxins have been reported, only those of the aflatoxin type appear to have been shown to be related to incidence of rainbow trout hepatoma. Therefore, data reported in this paper and the accompanying review are concerned in the main with aflatoxins, although reference is made to other toxins elaborated by *Aspergillus flavus*.

Since the chemistry, toxicity, pathology, production, occurrence, and measurement of aflatoxins is summarized in the review, this paper will deal with levels of aflatoxins reported in current and potential fish foods. Levels of aflatoxins in commodities presently under test are also reported.

It should be emphasized that the number of samples of each commodity listed in the table is far too small to warrant definite conclusions. Of equal or greater importance, the analytical methods available for measuring aflatoxins are at best semiquantitative. With these restrictions in mind, the

following published and unpublished data have been tabulated in table 60.

Both percentage and parts per billion are shown to emphasize the extremely small amounts being determined.

The results have been separated into aflatoxin levels in products contaminated naturally, and in feedstuffs in which aflatoxins have been induced by adding an *A. flavus* culture or some other mold that produces aflatoxins.

In grains, milo has been found to contain 5 to 30 parts per billion of aflatoxins. In the breakfast cereals the aflatoxins appear at relatively low levels. Additional analyses are needed to confirm or refute the presence of aflatoxins in breakfast cereals.

Among oilseeds and products, we find Sudanese cottonseed cake has been reported in a British publication to contain aflatoxins. The cake contained approximately 500 parts per billion of aflatoxins.

Values for peanuts ranged up to a thousand parts per billion. In work in which a culture was added, reports show up to over two and a half million parts per billion.

The peanut meal values ranged from five or less than 40 up to a high of 10,200 parts per billion. In some cases the individual components were reported as shown for the last meal in which there was 4,500 each of B_1 and G_1, and 600 each of B_2 and G_2.

TABLE 60.—*Aflatoxins in agricultural commodities*

Commodity	Produced				Reference in Phelps' Review
	Naturally		By added culture		
	%	ppb	%	ppb	
I. Animal byproducts					
Meat and bone meal	0–.000003	0–30	—	—	*
II. Grains					
Buckwheat	—	—	+	+	12
Corn or meal	—	—	+	+	12, 18, 21, 83
Maize	+	+	—	—	22A
Milo	.0000005–.000003	5–30	—	—	*
Rice	—	—	+	+	12
Rye	—	—	+	+	12
Wheat	—	—	+	+	12, 25, 47, 61, 69
	(B_1 only)				
III. Miscellaneous breakfast cereal					
A	trace	trace	—	—	*
B	.0000006	6	—	—	*
C	.000001	10	—	—	*
D	.0000016	16	—	—	*
E	.000001	10	—	—	*
F	.0000006	6	—	—	*
G	.0000008	8	—	—	*
H	.000002	20	—	—	*
		(+50 ppb G_1)			
Potatoes	—	—	+	+	47
IV. Oilseeds and products					
Cottonseed	—	—	+	+	
Cottonseed cake (Sudanese)	.00005	500**	—	—	52
Peanuts	.0001	1000	—	—	68
Peanuts	—	—	.014–.265	140,000–2,650,000	29
Peanut meal	.0000005–.00035	5–3500	—	—	59, 60
Peanut meal	<.000004–.0008	<40–8000	—	—	22
Peanut meal	.000002–.00044	20–4400	—	—	34
Peanut meal	.000002–.001	20–10,000	—	—	9
Peanut meal	.0002–.0005	2000–5000	—	—	
Peanut meal	.0006	6000	—	—	10
		(5000 B_1, 1000 G_1)			5b
Peanut meal	.0012	10,200	—	—	63
		(4500 B_1, 4500 G_1, 600 B_2, 600 G_2)			
Runner beans	—	—	+	+	69, 70
Soybeans	—	—	+	+	12, 69
Soybean meal	.0000015–.000003	15–30	—	—	*

* Data supplied by NCPA member company.

** Individual components determined by U.S. Laboratory (290 B_1, 104 B_2, 58 G_1, 29 G_2).

Soybean meal contained low levels in the one report available, 15 to 30 parts per billion.

Jack Coates mentioned this morning the values that had been found in the 16 experimental and commercial cottonseed meals that are being fed to trout at different locations around the country. These values are broken down in table 61, into the individual components as found by two methods. The FDA celite method used was a slightly modified form of the FDA celite method. As far as we know, it gives comparable results, but it is not exactly the same as the FDA celite method.

The meals were also analyzed by the Procter & Gamble Florisil method. I think there is generally good agreement that the P & G method is reading a little bit lower than the FDA celite method.

At the bottom of table 61 are shown aflatoxin analyses on those portions of the large shipments of meal used for preparing the lipid extracts of cottonseed meal fed by Dr. Halver. These small amounts of the five large shipments were extracted by the Bloor-

TABLE 61.—*Analyses of commercial and experimental cottonseed products fed in trout hepatoma study*

Cottonseed meal	Process*	Year seed produced	Aflatoxins (ppb) by method							
			Modified FDA Celite				P&G Florisil			
			B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
C	SP	1962	250	60	neg.	neg.	175	***		
F	SP	1962	200	15	neg.	neg.	175			
(77 ppb by Oregon State method)										
G	SP	1962	190	20	neg.	neg.	135			
P	SO	1963	65	75	neg.	neg.	45			
(257–343 ppb by SRL Pons-Goldblatt method on screened sample)										
K	SP	1963	25	neg.	neg.	neg.	25			
O	SO	1963	8	neg.	neg.	neg.	4			
E	SP	1962	4	neg.	neg.	neg.	8			
D	SP	1962	neg.	neg.	neg.	neg.	6			
A	PPS	1962	neg.	neg.	neg.	neg.	neg.			
B	PPS	1962	neg.	neg.	neg.	neg.	neg.			
H	SP	1963	neg.	neg.	neg.	neg.	neg.			
I	SO	1962	neg.	neg.	neg.	neg.	neg.			
J	SP	1963	neg.	neg.	neg.	neg.	neg.			
L	SO	1963	neg.	neg.	neg.	neg.	neg.			
M	PPS	1962 & 1963	neg.	neg.	neg.	neg.	neg.			
N	PPS	1963	neg.	neg.	neg.	neg.	neg.			
Cottonseed flour			neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

Meal batches used for lipid extracts**			SRL Pons-Goldblatt method			
NCPA code	Halver code	Meal type	B_1	B_2	G_1	G_2
C	2	SP	300	128	neg.	neg.
K	3	SP	28			
A	1	PPS	neg.			
I	4	SO	neg.			
J	5	SP	neg.			

* SP = Screwpressed, SO = Hexane extracted by commercial hexane, PPS = Screwpressed followed by hexane extraction.

** Extracts prepared by SRL, New Orleans, using modified Bloor technique.

*** No value reported.

TABLE 62.—*Summary of aflatoxin analyses vs. trout hepatoma*

Meal	Aflatoxins, ppb		Hepatomas at 300 days, %*
	B_1	B_1+B_2	
C	175–250	175–310	57
G	135–190	135–210	30
P	45– 65	45–140	25
F	175–200	175–215	18
H	negative	negative	4
K	25	25	0
O	4–8	4–8	0
E	4–8	4–8	0
D	negative	negative	0
A	----do----	----do----	0
B	----do----	----do----	0
I	----do----	----do----	0
J	----do----	----do----	0
L	----do----	----do----	0
M	----do----	----do----	0
N	----do----	----do----	0

* Ralston Purina data presented by Jack Coates

La Roche technique by Dr. Goldblatt's group and by Henry Vix's engineering group at the Southern Regional Laboratory. The results are almost identical with the comparable meals in the top part of table 61, but the samples were from different sacks within the shipment. There are slight differences and you can draw your own conclusions whether they are of any significance.

As Jack pointed out this morning, the incidence of rainbow trout hepatomas found in their work correlated well with meals showing the higher levels of aflatoxins, as summarized in table 62. More than half of the meals, however, were negative for aflatoxins.

In table 63 we see the results when part of Meal P that was used in the collaborative trout feeding test was sent to 11 laboratories. I have lumped together the values obtained by three aflatoxin thin layer chromatography methods. All of the laboratories analyzed this sample of Meal P by the recently developed Pons-Goldblatt aqueous-acetone, thin layer chromatography method, and some groups used, in addition, the FDA celite method and the Procter & Gamble Florisil method. For the sake of simplicity, they have all been lumped together. If any of you are interested, we would be glad to send you the complete tabulation.

I would like to point out that Laboratory 2 obtained a high of 560 parts per billion of B_1 from this sample of meal, and Laboratory 4 found in the 10 to 24 parts per billion range. Since then, Laboratory 4 has improved their technique a little. Since they found they were overloading their plates, they have now obtained a value of about 300 parts per billion. Part of the variation in results may be due to sampling, but every precaution was taken to finely grind the material and mix it by hand to eliminate sampling problems. I do not know how you can ever eliminate the problem completely.

This morning we were talking about feeding gossypol. I think we should have pointed out that the level mentioned was 10 to 15 times the level encountered in a practical-type ration. It is no criticism of the work, but I think we need to keep the levels in mind from time to time.

In summary, mold toxins, and more specifically, aflatoxins, appear to be contaminants in many agricultural commodities. Results available to date indicate that many species of domestic farm animals can withstand, without effect, levels of aflatoxins likely to be encountered in practical-type rations formulated with domestic commodities, but

TABLE 63.—*Results from aflatoxin analysis of meal P*

Laboratory	Aflatoxins, ppb			
	B_1	B_2	G_1	G_2
2	560	neg.	140	neg.
8	550	trace	neg.	neg.
1	360–475	235–285	neg.	neg.
11	350	neg.	60	neg.
10	260–290	125	neg.	neg.
5	84–220	30–104	neg.	neg.
3	140–143	70–120	neg.	neg.
7	140	29	14	neg.
9	130	21	neg.	5
6	109	32	30	15
4	10–24	neg.	trace	neg.

additional research is needed. Levels of aflatoxins reported for commodities listed in this paper should *not* be considered to be representative since only a very limited number of analyses are reported. It is also obvious from data shown in table 63 that either available methods for measuring aflatoxins warrant further refinements, or improvements are needed in sampling techniques.

DETECTION AND ELIMINATION OF AFLATOXIN

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ASSAY FOR AFLATOXIN

Two main groups of test methods have been proposed for determination of aflatoxin, one biological and the other chemical. The biological test methods include use of ducklings, chick embryos, microorganisms, enzymes, and cell mitosis. The chemical tests are based on specific chemical properties, especially fluorescence of purified aflatoxins.

The most widely used biological assay procedure is that based on development of liver lesions in ducklings. This test utilizes a relatively crude extract, affords a specific toxicological response, and can be made roughly quantitative but does not distinguish between the four related toxic components of the mixture commonly referred to as aflatoxin. Ducklings appear to be the animal species most sensitive to aflatoxin and the sensitivity decreases quite rapidly with age. Accordingly 1-day-old ducklings are generally used.

Numerous variations of the duckling test, originally proposed by Sargeant et al. [*1*] have been proposed and additional variations are under development. A procedure recently reported by Armbrecht et al. [*2*, *3*] is illustrative. Essentially, this procedure provides for the preparation of a suitable extract and administering the partially purified extract (dissolved in propylene glycol) orally to day-old Peking White ducklings by the crop-tube method. For qualitative screening each bird of a pair is given 0.5 ml of test solution and the volume is increased 0.1 ml each succeeding day for a total of 7 days. The ducklings are sacrificed and the livers examined for color and other gross pathologic features and then, after preparation of sections, examined microscopically. For quantitative measurements an LD_{50} determination is recommended.

The biological test in use in the United Kingdom is the recommended procedure of the Interdepartmental Working Party on Groundnut Toxicity. In this test, the equivalent of 80 g of meal is administered in the form of an extract to each day-old duckling over a period of 4 days. Observations are made for characteristic liver lesions. Details of the test may be obtained from the Director of the Central Veterinary Laboratory, New Haw, Weybridge, Surrey.

A biological assay procedure for aflatoxin using fertile chicken eggs was reported by Platt et al. [*4*] who observed that the quantities of "groundnut toxin" required to cause chicken embryo deaths were about 1/200 of those required for a positive result with day-old ducklings. Although this test is considerably more sensitive than the duckling test it appears to suffer from poor reproducibility and nonspecificity. More recently the possibility of using the chick embryo in the assay of aflatoxin toxicity was investigated by Verrett et al. [*5*] and "was found to be feasible." Injections of aflatoxins in purified extracts dissolved in propylene glycol are made before incubation, in fertile White Leghorn eggs, by either of two routes: yolk

or air cell. Groups of at least 20 eggs are used at each dose level of a sample. The LD_{50}'s reported were 0.048 and 0.025 micrograms of aflatoxin B_1 for the yolk and air cell routes respectively. This compares with about 2.0 micrograms of aflatoxin B_1 required to produce minimal bile duct hyperphasia for day-old ducklings [*6*].

Attempts to develop a microbiological assay for aflatoxin appear thus far to have been unsuccessful. Thus it is reported [*7*] that "microbiological screening showed that aflatoxin did not inhibit the growth of certain standard organisms frequently used in this type of assay." However, it is known that several groups are currently pursuing this approach [*8*].

The possibility of using enzyme systems for assaying *A. flavus* toxins, e.g. by observation of rate of incorporation of [^{14}C] leucine into protein by various liver preparations *in vitro* has been discussed by Smith [*9*] but the technique does not appear to have been developed further. Other biological assay methods which have been considered include immunological approaches and the effect of aflatoxin on mitotic division in cultured embryonic lung cells [*10*]. In the method described, as little as 0.01 microgram of aflatoxin could be detected within 24 hours. Evidence seems to be accumulating that aflatoxins are inhibitors of protein synthesis (Black and Altschul, 1965). The primary objective of most of these investigations is to develop basic information on physiological aspects of response to aflatoxins but results such as those reported may contribute to methodology for assay of aflatoxin.

Numerous different chemical analytical procedures for determination of aflatoxin have been developed and chemical assay methods are in a state of flux. Although differing greatly in specific details (and it appears almost as though each laboratory has introduced individual innovations) nevertheless the basic patterns of most methods are substantially identical. Basically these general procedures consist of sample extraction with a solvent, more or less elaborate but still only partial purification of the extract containing the aflatoxin followed by some chromatographic procedure to separate individual aflatoxin components in a form and purity adequate for evaluation of the intensity of fluorescence of the components when illuminated with longwave ultraviolet radiation [3660 Å].

Until quite recently attention for the most part had been devoted to assay for aflatoxin in peanuts and peanut products. The basis for the extraction solvent had been laid quite early by Allcroft [*11*] who reported "A toxic preparation was obtained by a chloroform extraction—of a dispersion in water of the material isolated from the meal by a methanol extraction." That is, the initial extraction was with methanol and partial purification was achieved by various treatments followed by separation into chloroform. In all cases prolonged Soxhlet type extraction was used. Different type extraction solvents were apparently first introduced in 1964. These newly introduced solvents included methylene chloride [*8*] acetone-hexane-water for peanut products [*12*] and aqueous acetone for cottonseed products [*13*]. Introduction of more rapid extraction procedures, e.g. by use of the Waring Blendor [*14*] and mechanical agitation [*13*] materially facilitated chemical analyses for aflatoxin. Elimination of the Soxhlet type extraction alone shortened the time of analysis by many hours.

Initially, separation of the partially purified aflatoxin components was achieved by paper chromatography

[*15*]. The solvent systems employed for development of the paper chromatograms typically required from 3 to 6 hours for separation of aflatoxin components. Subsequently thin layer chromatography (TLC) was employed, at first using alumina as the adsorbent [*16*] and later silica gel in the determinative step. At present TLC using some form of silica gel, e.g. Silica Gel G or Silica Gel G-HR seems to be the preferred adsorbent for TLC. The amount of the different aflatoxins is estimated by comparison with a standard of the intensity of the ultraviolet induced fluorescence of spots of appropriate R_f, by estimation of the dilution to lowest observable fluorescence, or by elution and estimation of the intensity of fluorescence of the eluate in a fluorescence spectrophotometer. An excellent comparison of several procedures which were employed in the autumn of 1963 for assay of aflatoxin in peanut products by laboratories concerned with the problem was recently published by Trager et al. [*17*] but as the authors noted "even while this comparison was being made, all the laboratories were in the process of modifying their procedures." They are still modifying them.

More recently at the Southern Regional Research Laboratory we have developed what might be called micro or semi-micro methods which permit analysis of individual peanut kernels, or even a small part of a single kernel, for aflatoxin. Using this technique we have found individual kernels containing upwards of 100,000 parts per billion of aflatoxin, indeed even in excess of a million parts per billion. Further, shavings of various parts of a peanut kernel could be analyzed permitting the mapping of the concentration of aflatoxin throughout the kernel (Cucullu et al., 1965).

Although other physical methods such as gas liquid chromatography and ultraviolet and infrared spectrophotometry could be used for analysis for aflatoxin these methods are not as sensitive as those based on fluorescence and do not appear to be used for the analysis for aflatoxin in food and feed products. However another physical method that does appear to be sufficiently sensitive and to have possibilities is based on their polarographic characteristics [*18*]. Thus far the technique has been used successfully only with relatively pure samples of aflatoxin.

SOME APPROACHES TO THE ELIMINATION OF AFLATOXIN

The first and perhaps the best approach to minimize contamination of foods and feeds with aflatoxin is prevention. Sound, healthy seed appears to be more resistant to mold growth and production of aflatoxin than is damaged seed. Consequently suitable harvesting and handling procedures to avoid mechanical damage are essential. This may necessitate slowing down, or redesigning machinery or equipment now used in lifting, windrowing, combining, conveying, and drying peanuts prior to storage to minimize mechanical damage. In the case of cottonseed, operational changes might be required in mechanical harvesting, ginning and conveying cottonseed prior to storage. Research sponsored by the Department is currently underway directed to development of improved equipment and practices to reduce mechanical damage to cottonseed and peanuts (M.S. Agr. Res. Serv. 1965).

Moisture content is a critical factor. It is known that relatively high temperature and humidity afford conditions conducive to mold growth and production of aflatoxin. Reduction of moisture below the critical level necessary for germination of spores is probably the

best single means of controlling mold development and production of aflatoxin. Adequate storage facilities to maintain a low moisture level and to prevent opportunity for mold growth are required. It is difficult to assign precise safe moisture levels for materials in process or in storage. Because the effects of moisture and temperature are interrelated, because of the effect of damage and because of different oil contents of mature and immature seed as well as of different types of seed. Thus at 60° F. there was no increase in the free fatty acid content of prime cottonseed even after 4 months' storage at 16 percent moisture while at 90° F. the free fatty acid content of prime seed with the same moisture content increased rapidly after about 40 days' storage and rose to about 10 percent FFA after 4 months. This increase was even more pronounced at higher moisture contents. In the case of slightly damaged seed with 16 percent moisture storage even at 60° F. resulted in an initial slow rise in FFA and a rapid increase after about 20 days [*19*]. Immature seed contains less oil than does mature seed and this, too, is a factor. For example, the safe moisture level for peanuts is generally accepted as being lower than that for cottonseed. However, the oil content of shelled peanuts is about 50 percent whereas the oil content of cottonseed is about 20 percent. If these materials are compared on an oil-free basis (considered appropriate because water is not associated with the oil) the "safe" moisture levels become more nearly equal.

It should be noted, furthermore, that this "safe" level is the moisture content of the individual kernels or seed and not the *average* moisture content. Uneven moisture distribution among kernels may result in serious mold growth even when the average moisture content would appear to be at a safe level. Mold produces moisture as it grows, so once fungal growth has started in one excessively wet kernel the moisture content of the immediately adjacent kernels also increases and further fungal proliferation may proceed regardless of average moisture content. This will result in a pocket of highly contaminated material. It will be recalled that our experience is that at least in the case of peanuts the vast majority of aflatoxin occurs in a relatively very small number of highly contaminated kernels.

This fortuitous circumstance that the vast majority of the aflatoxin in contaminated materials may reside in a relatively small number of kernels may afford an opportunity for mechanical methods for effectively reducing the aflatoxin content of selected materials by separation of seed that may have become contaminated with aflatoxin despite efforts at control. Thus in the case of peanuts culling on the basis of physical characteristics such as size (including immatures), obvious damage, and discoloration appears to offer a feasible means of removal of at least a major part of any peanuts that may be highly contaminated with aflatoxin. The peanut industry has recognized this as one of the possible means for decontamination and has taken steps for improved mechanical separation. Electronic sorters have been used advantageously. Additional segregation after splitting may be necessary to reduce still further aflatoxin content of edible grade peanuts, especially for peanuts with hidden mold. In the case of cottonseed, segregation on the basis of density may be of value. At the Southern Utilization Research and Development Division (SU) projection devices (such as the ARS Differentiator) have been developed and operated on a pilot-plant scale to separate trash from cottonseed [*20*]. In

this work it was found that these devices also graded the cottonseed according to density. The sound and mature cottonseed were projected relatively long distances (30 to 90 feet) whereas immature cottonseed with a high free fatty acid content fell short. Since, at least in the case of peanuts, there is a high correlation between fat acidity and the percentage of samples containing aflatoxin [*21*] this may afford a means of mechanically removing the major portion of aflatoxin-contaminated cottonseed.

Removal of aflatoxin by means of solvents when processing cottonseed and peanuts to oil and meal seems to offer excellent potential. Aflatoxin that accompanies the oil obtained from processing of seed (whether by mechanical expression or by solvent extraction) presents no special problem because the aflatoxin can be subsequently removed from the oil in conventional refining and bleaching operations. Current processing practices, either mechanical pressing or extraction with commercial hexane, leave in the residual meal the vast majority of aflatoxin that may have been present in the seed. However, processes using selective solvents which remove aflatoxin have been developed at SU. For plants where solvent extraction with commercial hexane is now used, replacement of the hexane with a mixture of acetone, hexane, and water appears to offer promise. This solvent system which was originally investigated on a pilot-plant scale at SU to remove gossypol along with oil from prepared cottonseed meats leaving a highly nutritious cottonseed meal [*22*] also extracts aflatoxin with the oil and gossypol. The aflatoxin and gossypol can, of course, be readily removed from the oil by refining and bleaching. Use of such a solvent mixture would require relatively little change in existing direct hexane extraction plants. In the case of peanuts this solvent mixture has been used on a pilot-plant scale with prepressed peanuts and with peanut meals to remove more than 90 percent of the aflatoxin present.

Another solvent system that appears to offer promise but has not yet been evaluated on a pilot-plant scale is acetone containing a small proportion of water. This, like the mixture of acetone, hexane, and water, is also an excellent solvent for removal of oil, gossypol, and aflatoxin from prepared cottonseed. This solvent system could be used to remove the residual aflatoxin and gossypol from the extracted meal produced in mills processing cottonseed either by mechanical pressing or solvent extraction with hexane.

Still another approach is offered by a solvent such as 70 percent aqueous acetone. This is the solvent currently used for the analysis of gossypol, in which it is quite soluble. This solvent also readily dissolves aflatoxin and free fatty acids but dissolves relatively little neutral oil and other desirable nutrients from prepared cottonseed. The extracted residue is in a form suitable for preparation of a full-fat cottonseed flour or for defatting to a predetermined lipid content by mechanical means or by a conventional solvent such as hexane. In addition, the oil produced is substantially equivalent to a refined oil.

Numerous treatments to destroy or inactivate aflatoxin have been proposed but relatively little will be found in the recorded literature and much of what is reported is sketchy and contradictory. For example, Blount [*23*] reported that "when the groundnut meal was heated to a temperature of 150° C. for 1 hour this appeared to make the product even more toxic" (for turkey

poults), but Allcroft [*11*] reported "our tests showed that toxicity of the meal to ducklings was always less after it had been heated." Again such statements may be found as "Steam heating or other processes do not inactivate the toxins. When toxic meal was heated to 150° C. for several hours the protein was denatured but the toxicity was not reduced" [*24*]. Recent reports [*8*] indicate that moist heat treatment of partially purified aflatoxin at a temperature of 170° C. and a pressure of about 80 to 90 psi for 10 to 20 minutes destroys the aflatoxin as judged by chemical assay and the toxicity as judged by chick embryo tests. We have found that this type of treatment applied to peanut meal eliminates the chemical response for aflatoxin but produces other materials with characteristics very similar to those of aflatoxin with respect to behavior during purification and TLC.

Destruction of more or less purified aflatoxin by various chemicals including chlorine, hypochlorite, sulfur dioxide, alkalies, and acids has been suggested [*25*] as well as chlorine dioxide, hydrogen peroxide, and potassium permanganate, etc., [*8*] but such treatments may not necessarily be adequate when applied to cottonseed or peanuts and products prepared from these oilseeds. The effectiveness of 1 percent $CaCl_2$ solution as an extractant to remove aflatoxin from peanut meal has been reported [*26*]. The consensus seems to be that chemical means for detoxification of cottonseed and peanut meals presents some major problems. Treatment adequate for destruction of aflatoxin could seriously impair the nutritive and other properties of both cottonseed and peanut meal. Nevertheless such reports are very sketchy and we at SU are conducting tests on chemical detoxification, especially with gaseous reagents such as ammonia, or with reagents that may be added during processing. The intent here is to provide maximum opportunity for prolonged and intimate contact so that the aflatoxin may be inactivated and the toxic effects may be eliminated while using relatively mild chemical reagents. Such treatments to be effective must also be feasible from other standpoints such as ease of introduction into currently used processes, economy of operation, and maintenance of nutritive quality.

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Discussion

DR. GOLDBLATT: As Dr. Newberne indicated, I was really pleased when Dr. Wogan showed a slide that a poor, lonesome, organic chemist could understand at long last. But I thought after seeing all those slides of livers, you ought to have the opportunity to at least see one thin layer chromatographic plate.

Figure 103 was not made for this purpose, and actually, although it may sound strange, I just brought it along by accident, because I thought others were certainly going to show slides like this.

In the middle are two chromatograms of a standard solution of fairly pure aflatoxin. On the right are chromatograms of 1, 3, and I believe 5 microliters of a test solution obtained after rather extensive purification of a methanol extract of a peanut meal. On the left are chromatograms of 1, 2, 5, and 10 microliters of a test solution obtained after a fairly simple purification of an acetone-hexane-water extract of the same peanut meal. You will note there is much more extensive streaking in the chromatograms on the right than in those on the left. The chromatograms of the standards in the middle show good separation of aflatoxins B and G. Aflatoxin B, of course, is the upper spot and G is the lower spot. Knowing the amount of each aflatoxin present in the

FIGURE 103.—Thin layer chromatograms of extracts obtained with acetone-hexane-water solvent (left) and methanol (right).

two chromatograms of the standard one estimates the amount of aflatoxin in the sample by comparing the intensity of the spots of appropriate R_f in the chromatograms of the sample extract with those in the standard. Obviously such a comparison would be easier with the chromatograms on the left. The purpose of the slide originally was to show what can be done in the way of improving the cleanness of a plate, and hence in the ease of estimating how much aflatoxin there is present in the sample simply by changing the extraction solvent.

I want to emphasize that chemical methodology is in a state of flux. Many people are working in the area; a lot of progress is being made, as Dr. Phelps indicated, and analytical methodology is being improved all the time.

Basically, practically all the chemical procedures are using the method that was indicated. That is, the procedures involve first an extraction, a purification step, application of some of the purified, concentrated extract to a thin layer chromatographic plate, development and evaluation of the intensity of the fluorescence of spots of appropriate R_f.

Different people are using different extracting solvents, different modes of purification, different TLC coatings and different developing solvents.

The vast majority of people are still using visual methods for evaluation. The visual method may be by comparison of intensity of spots with those of some standard or by dilution down to where you just barely can see them. But increasingly, objective methods are being introduced, for example, densitometry.

MR. SINNHUBER: We have been using a method quite similar to the one Dr. Goldblatt is using. We are using a hexane extraction to remove lipid, and then acetone, U.S.P. grade acetone, to extract aflatoxin. At the same time we obtain some residual lipid pigments.

This extract is then cooled overnight to precipitate out the lipid and pigments. The solution is then filtered and the filtrate evaporated and taken up in methanol. Then it's cooled again and by the same procedure filtered in order to remove additional insolubles and interfering pigments.

Most all this work has been done with cottonseed meal. We were trying to devise a method for cottonseed. The usual procedure for peanuts was not suitable, in our hands at least.

Then the methanol filtrate was diluted with water and extracted with chloroform using a separatory funnel. The chloroform extract is concentrated and ready for spotting on thin layer plates.

We have been quite successful in assaying cottonseed meal. Dr. Goldblatt last week mentioned a clean-up procedure, which we have been using very successfully to remove some highly pigmented cottonseed meals or meals that have been mishandled.

We are now using fluorescent densitometry for reading our thin layer plates. It is a new instrument and we believe it will be useful. We have been able to read on a thin layer plate by this method a spot containing 2.5×10^{-4} micrograms. This is, I believe, several times more sensitive and certainly more accurate than the naked eye. Standards are compared at the same time.

DR. GOLDBLATT: That is one of the things I wanted to indicate, the increasing tendency to make the assays or the evaluations objective. As I see it, the subjective assays that we are doing now are no more accurate than one significant figure, at the very, very most two significant figures. Values like 1.43 are nonsense. It's really accurate to only one significant figure.

As I indicated, the chemical methods of analysis are in a state of flux, but even so, attempts are being made to standardize them. At the present time it seems almost as though every research laboratory is using its own variation or modification. But I would call your attention to the fact that groups are now being organized to try to get some degree of standardization.

A committee of the American Oil Chemists' Society has now been formed for the analysis of aflatoxin; also a committee has been formed by the AOAC. That's the Association of Official Agricultural Chemists. And there is now a joint committee of the two to try to get the two groups together to see if we can adopt a single uniform set of methods. So, even though it may be premature to actually formulate a set of standard methods of analysis for various commodities it probably isn't premature at least to start working toward a standard method of analysis. And it could well be that we will have a different accepted standard method of analysis for each commodity or for different commodities or different parts of a commodity. For example, one method might be used for peanut meals and another for peanut butter.

I would like to show you a few more slides that deal with work that we have done in the Southern Laboratory, and what I refer to as micro methods or semi micro methods of analysis.

We were interested in being able to analyze a single peanut or half of a peanut for aflatoxin content. This particular half peanut shown in figure 104 was selected for the reason that it shows very nicely these black spots that are characteristic sclerotia of an actively

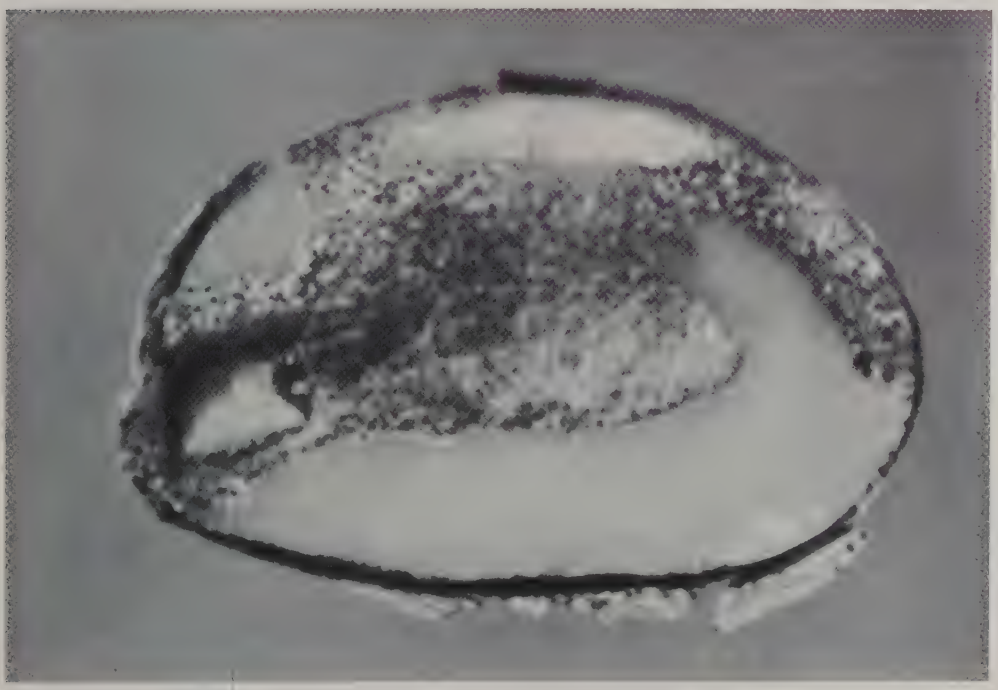

FIGURE 104.—Half of an infected peanut kernel showing sclerotia.

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growing mold or of a mold that almost certainly will produce aflatoxin. Many people feel that those strains of *A. flavus* that produce those sclerotia, those little black spots, will produce aflatoxin. As you know, many strains of *A. flavus* are not known to produce aflatoxin. Some do.

The other half of this particular peanut had about 30,000 parts per billion of aflatoxin in it. On the outside this looked like a perfectly normal peanut. It would have passed an inspector. But when it is cut you see this kind of thing. So, if an inspector had examined the split peanut, there would be just no doubt that it would have been picked out or rejected.

Figure 105 shows a half of another peanut of the same kind. From the outside this peanut looked normal. The other half of this peanut had 100,000 parts per billion of aflatoxin in it. Different parts of this half of the peanut (shown in fig. 105) were cut out and analyzed separately. Interestingly enough, the black portion in the middle analyzed only about 10,000 parts per billion of aflatoxin, the germ, shown at the right, contained about 27,000 ppb; a strip from the abaxial surface below the germ contained 98,000 ppb, some white mesophyll above it contained 130,000 and a strip at the adaxial surface contained 340,000 ppb of aflatoxin.

Now, the significance of that to me is this: aflatoxin is spread out throughout the peanut. And I think the hope that we had a year or so ago to the effect that it might be possible to remove aflatoxin by a mechanical wash has to be rejected, at least for peanuts of this kind. You just couldn't hope to wash out the aflatoxin from a peanut like that.

FIGURE 105.—Half of a peanut kernel the other half of which contained 100,000 ppb of aflatoxin.

It's not on the surface. You have to cull out the whole peanut.

Incidentally, this half of the peanut represents one half of each of the two cotyledons; that is, this is not one peanut split into the two cotyledons. It contains one half of each cotyledon. And in part, this black material in the middle acts as a cementing material between the two cotyledons.

We have also taken some peanuts and analyzed the two cotyledons separately. The two cotyledons don't necessarily have the same amount of aflatoxin in them. So it's not uniformly distributed between either the two cotyledons or along the surface between the two cotyledons.

The next slide (fig. 106) is an interesting one, I think, for the reason that any inspector just looking at the peanut without splitting it would have passed it and it would not have been rejected. If you split it open you can see some darkening near the middle but no where nearly as bad as the previous peanut. But the other half of this peanut analyzed nearly a million ppb of aflatoxin. On the outside it looked normal; on the inside, you wouldn't use it but it didn't look as bad as the two that you saw just before. Interestingly enough, in this case we did not find any aflatoxin in the germ. A strip of the abaxial surface showed 130,000 ppb and a piece of the white mesophyll contained 18,000 ppb but a piece of the orange spot near the center was found to contain 2 million ppb of aflatoxin.

Now, the point I want to emphasize is the terrific concentration that you may have in certain individual peanuts.

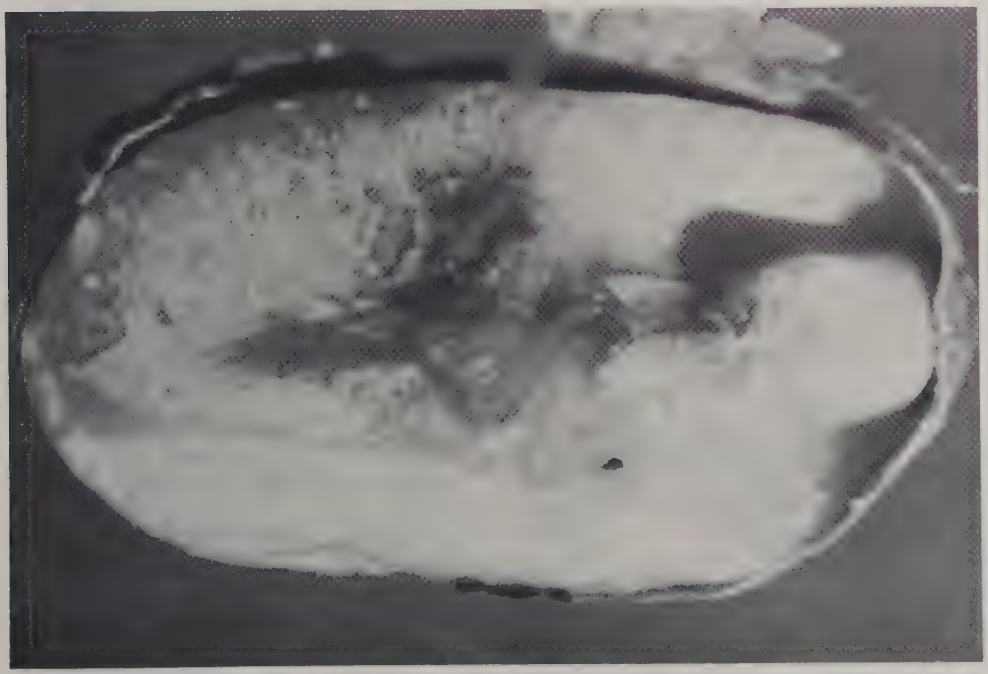

FIGURE 106.—Half of a peanut kernel, the other half of which contained nearly a million ppb of aflatoxin.

If you had just one of those peanuts in a million you would still have one part per billion of aflatoxin, or if you had one in 10,000 you would still have a hundred parts per billion in that whole 10,000. The contamination is extremely —or can be extremely—selective.

So the sampling problem is an extremely difficult one. If you started with a sample of 10,000 peanuts (that is roughly 5 Kg) and the sample contained this one peanut and the other 9,999 contained none, the batch would assay 100,000 ppb. Yet if this one peanut was missed in collecting the sample the batch would assay negative.

I want also to call attention to a potential new method of analysis, that we might call a biochemical method of analysis, that I did not refer to in the workbook. This was described in an article that appeared in the January 2, 1965, issue of Nature by Schoental and White entitled "Aflatoxins and 'Albinism' in Plants." It's on pages 57 and 58 of that issue. It represents what is to me a new method, or a new potential method, of analysis for aflatoxin. They use seeds of plants to detect and estimate the amount of aflatoxin. I would like to read just a few sentences from that article:

"For the test, twenty seeds of cress, *Lepidium sativum* L., were explanted on circles of surgical gauze placed in 2-in. Petri dishes containing 4 ml. of distilled water (controls) or of aqueous solutions, or suspensions, of the substances being tested. * * * Aflatoxin(s), like coumarin" (the authors tested about half a dozen additional compounds other than aflatoxin, and coumarin is one of them) "inhibited the germination of seeds in concentration of 25 μg/ml., but its effect on the colour of leaves was more marked. Complete absence of the green colour occurred in the presence of 10 μg/ml. of aflatoxin, and partial inhibition, with a characteristic bleaching of the edges of leaves, could still be recognized at 1 μg/ml."

Now, that's a relatively simple and relatively sensitive method of detection of aflatoxin. Coumarin does the same kind of thing, and that has that characteristic lactone ring that we have observed in the aflatoxins. It occurred to me that the two compounds, 2 and 3, that Dr. Wogan referred to on one of his slides would also be of interest for use in this kind of test. Possibly they could show up that same kind of behavior and indicate the presence of a coumarin type structure. I would like to add that to the list of possible biochemical methods that one could use for detection or estimation of aflatoxin. It's inexpensive; all you need is some cress seed.

I have another topic. That is detoxification or removal of aflatoxins. As I see it, there are really three approaches. The first and probably the best approach, as I indicated in the workbook, is prevention. Do the best you can to keep things dry. Don't let them get contaminated with mold, and so on.

The second method is removal, either mechanical removal of contaminated peanuts or cottonseed, or what have you, by any physical means that is available, or by solvent extraction. We think it is feasible to remove aflatoxin by solvent extraction.

The third method is chemical treatment, chemically altering the aflatoxin that is present.

Several methods have been investigated. I think we are becoming more optimistic about the possibility of chemically deactivating aflatoxin. I think at the M.I.T. meeting a year ago people were very pessimistic about the possibility of destroying aflatoxin.

A chemical treatment that we think is at least not unfeasible was worked

out jointly between the Southern Laboratory and the Western Laboratory. Dr. DeEds gave me some relevant data just this morning. The data were obtained on a peanut meal that had 700 parts per billion of aflatoxin treated to bring the aflatoxin content down to 17, 25, and 200 ppb.

The method that was used to evaluate this meal is the method that was developed by Dr. Masri at the Western Laboratory, and is another biological method. Basically it depends on the amount of gain of ducklings over a 2-week period; gain over a 1-week period might also be significant.

The weight gain I am reporting is in grams, for seven or eight ducklings. The gain for the ducklings on the control diet was 341 grams as the average gain over a two-week period. There is a beautiful progression as the aflatoxin content increases. For the original untreated meal, the gain was 149 grams. Treated, I will say mildly, so that 200 ppb were left, the gain was 214 grams; with 25 parts left, the gain was 232 grams; for 17 parts left, the gain was 272 grams. That is not as high as for the control.

We might say that final product apparently does not have as good nutritive value. Whether that is because of the 17 parts per billion of aflatoxin or whether we have damaged the nutritive value of the meal in the process, we don't know at this time. This is very, very recent data and it's really raw data, as you see. But I don't think we need be as pessimistic as we probably were a year ago about the prospects of, on the one hand, removal of aflatoxin; on the other hand, of chemical inactivation.

DR. NEWBERNE: On the peanuts you showed, in the slices away from the surface, how did the aflatoxin get in there? Was there mycelia rolling down into the nut?

DR. GOLDBLATT: I do not know. We are trying to follow that now. Grossly we couldn't see actual mycelia inside the cotyledon itself and we didn't examine this microscopically. The mass of mycelia is in that black stuff in between the cotyledons. That is really mycelia. But to me rather surprisingly, when we analyzed the black stuff, it was relatively low in aflatoxin content, *relatively* that is.

DR. PHELPS: You pointed out that an inspector would probably pass those peanuts. Would he pass them if they were viewed under ultraviolet light?

DR. GOLDBLATT: Yes. You can't distinguish anything with ultraviolet, and I should have qualified that. The inspector a year ago would have passed those peanuts. I think inspection procedures are different now than they were a year ago and I should qualify it in that way. I do not know whether an inspector today would have passed those peanuts or not.

DR. KUMMEROW: You said solvent extraction would remove aflatoxin. Does that mean that oil from solvent-extracted moldy peanut meal would contain the aflatoxin?

DR. GOLDBLATT: If you used the right solvent. The solvent that is used universally today in this country for extraction of cottonseed or peanuts is a petroleum fraction. For all practical purposes you can call it hexane. That will not remove aflatoxin, but there are at least three solvents that I would mention that do. One is this mixture of acetone, hexane, and water. It is not necessarily the azeotrope of acetone, hexane, and water, but a mixture of acetone, hexane, and water, including the azeotrope, will satisfactorily remove it; or aqueous acetone with just a little water in it will also remove aflatoxin.

In our own experience acetone extracts aflatoxin, but if you add a little

water to it, it's better. A mixture of about 98 percent acetone and 2 percent water would be more efficient for extraction of aflatoxin. At least in our hands that is the case.

Now, that will also remove oil; if you want to remove oil, that is fine. Or 70 percent aqueous acetone will effectively remove aflatoxin without removing oil, at least without removing any significant amount of oil. Traces of oil are removed. So, depending on what you want to do, you may use several different solvents.

The aflatoxin in any pressed peanuts would still very largely be in the meal. There is some that goes into the oil.

Well, any edible grade oil today, I would say, would not contain aflatoxin, because as various people have indicated, it's removed in the alkali refining, or the vast majority is removed during the alkali refining, and the last traces are removed during bleaching. And certainly all edible cottonseed oil—is refined and bleached.

I believe all peanut oil produced in this country today is refined and bleached. That was not necessarily the case ten years ago.

DR. SIMMONS: Corn oil is refined and bleached, and being bleached more than formerly in this country.

DR. GOLDBLATT: I don't know about this country, but in foreign countries the only oil that I would be aware of that might not be refined and bleached is olive oil. Is all olive oil bleached and refined?

That's the only edible oil that I am aware of that is not refined.

DR. ASHLEY: I wonder if you have checked the stage of development of *Aspergillus* in those peanuts? It looked to me like perhaps the mold would be in a quiescent or postproductive state with respect to aflatoxin, which would have perhaps allowed plenty of time for the aflatoxin to diffuse out into the flesh of the cotyledon. Has any competent micologist traced the growth and possibly correlated the stages of growth of the mold with the productive phases for aflatoxin?

DR. WOGAN: This point has been looked at in a number of situations, and it appears that aflatoxins are not produced during the active growth phase of the mold; that is, as the mycelia are accumulating there is almost no aflatoxin production, and when the growth levels—or the rate of mycelia growth levels off, at this point, then, the secretion of aflatoxin in the medium actually begins.

POTENTIAL HEPATOCARCINOGENS FOR FISH

By Hans L. Falk
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Although most of the evidence regarding the etiology of hepatocarcinogenesis in the rainbow trout points at the aflatoxins, a few other potential carcinogens ought to be considered. These findings of several investigators showed hepatocarcinogenic activity when aflatoxins were not detected or could not be expected to be present.

The specificity of the disease for rainbow trout, while not affecting closely related fishes like the brook or brown trout, but on the other hand producing similar cancers in certain strains of duck and rodents, awaits an explanation, if the aflatoxins alone are to be considered the responsible agents. Findings that the pathological changes produced in ducklings on feeding the hepatocarcinogenic fish diet are not identical with those on aflatoxin feeding may, of course, have other explanations since the fish diet may not be adequate for the duckling and may not suggest that a different carcinogen is present. In this connection, an answer is also needed for the observation that a highly hepatocarcinogenic diet containing 15 percent cottonseed meal became practically inert when liver was added to the diet without changing the content of cottonseed meal. Such a protective effect of dietary liver should be examined experimentally in relation to aflatoxin carcinogenicity.

The hepatocarcinogenicity of herring oil, which was established in earlier experiments, may also require a different explanation because of the ease of destruction of aflatoxin, which would most likely not be present any longer in the finished product if it was present at all in the original fish meal.

The analysis of the diet for aflatoxins showed that cottonseed meal may contain sufficient quantities of aflatoxin to account for the hepatocarcinogenicity, although not all cottonseed meals contained aflatoxins, while the diet produced hepatomas. Physical analysis of the active diet, as well as fractionation procedures, pointed at discrepancies: the R_f value and the fluorescence spectrum did not agree with the expected characteristics of aflatoxins, nor was the activity in the alpha fraction of the total lipids the fraction in which the aflatoxin was to be expected.

Using pure Aflatoxin B_1, it could be shown that in the presence of neutral fats the compound would dissolve in hexane and would show a fluorescence spectrum with a maximum at 450 $m\mu$. When shaken with aqueous methanol, the spectrum of Aflatoxin B_1, with a fluorescence maximum at 430 $m\mu$, would be detected in the aqueous methanol solution. It appears likely that even the R_f value may be altered by the presence of interfering substances, so that the above mentioned discrepancies may not be significant at all.

The stability of aflatoxins under experimental conditions was found to be very small and it was expected that some of the aflatoxin in the diet may have been altered. On exposure to artificial light, different products can be

detected on paper chromatography, which possess different R_f values with fluorescence spectra not far different from the parent substance. Nothing is known at the moment about the toxic and hepatocarcinogenic properties of these products.

Besides the aflatoxins, total fats of the fish diet contain a number of interesting compounds with potentially carcinogenic chemical groups, which may become more numerous after processing, handling or storing. Many toxic components are present in the fraction generally described as the non-urea-adduct fraction, which, however, proved too toxic to be adequately tested by feeding experiments on fish. By injection into rodents, non-urea-adduct fractions appear sarcomagenic. Dr. R. Sims will have described the detection and quantitation of ketones, hydroperoxides, epoxides and lactones in the hepatocarcinogenic diet. Evidence of carcinogenic activity for these groups of chemicals is still being collected. Peroxidation products of linoleic acid, however, are known to be hepatotoxic and hydroperoxides were shown to be carcinogenic to mice on painting and injection of certain specific compounds. Gamma-lactones of fatty acids have also been found sarcomagenic on injection into rodents, and epoxides of long chain fatty acids were leukemigenic to mice. The observation of lymphosarcomas in fish in earlier, as well as recent, publications is of interest in this connection.

Other dietary contaminants which may act as hepatocarcinogens, such as chlorinated pesticides, have been considered, but are of low order of magnitudes as contributory factors in this situation. The suggestion that a different kind of dietary influence may be responsible for the observed hepatomas was proposed by Ghittino and Ceretto, who described liver lipoid degeneration, followed by regenerative hyperplasia and neoplasia, as specific for the rainbow trout. The possibility that viruses were the causative agents was also considered, but no evidence could be obtained by the investigators to substantiate this suggestion.

I would like to divide the time for my presentation into two separate portions, one before Dr. Sims' presentation, the other thereafter. The reason for dividing it is that I have two different problems I would like to discuss, which have little to do with each other.

The first one deals with the possibility that in the toxic fish feed there may be other potentially carcinogenic agents that would not fall into the category of aflatoxins, but may be part of the general lipid composition or possibly lipid decomposition. The types of compounds I have in mind had been found to be carcinogenic in rodents, but they had never been too clearly defined; they had never been exhaustively tested, and their carcinogenicity had only been sporadically established. Much information is still missing regarding the dose levels, the route, the species, the vehicle and other salient facts for most of the compounds under discussion.

The compounds can be divided into certain chemical groups: the epoxides, with several well-established carcinogens among them; the hydroperoxides, a group with few carcinogenic, but many mutagenic members; and the lactones, where beta and unsaturated gamma lactones are carcinogenic. In each group there are many noncarcinogenic compounds, and therefore a clear-cut statement regarding the likelihood of carcinogenic activity of each group is impossible to propose. However, it was shown some time ago that epoxystearate, for instance, on injection into mice would produce sarcomas and that other epoxides would produce sar-

comas or carcinomas [*1,2*], while other epoxides again would produce lymphomas [*1*]. The whole group of epoxides has been suspect, although several reports indicate that we are not dealing here with a well understood series of carcinogenic compounds [*3*].

Some of the epoxides and diepoxides with low molecular weight, like butadiene diepoxide, have been shown to be carcinogenic by several investigators [*4*], but structural requirements for biological activity have not been worked out for the epoxides.

The second group of compounds, the hydroperoxides, have been suspected of carcinogenic activity, although the literature does not contain many valid reports of their carcinogenicity [*1,2*]. The likelihood that carcinogenic peroxides may remain in the lipid material after the processing and handling of the feed is unlikely. One can only suggest that where there is a chance that hydroperoxides are present, some of these may be carcinogenic, but one cannot be more specific at this time.

The third group is that of the lactones, which have also been described as carcinogenic, and lately certain structural requirements for carcinogenic activity have been somewhat pinpointed [*5*].

Until recently, it seemed that to possess carcinogenic activity, a compound had to belong either to the beta-lactones or certain unsaturated gamma-lactones. The double bond had to be in a certain position or the compound would not possess carcinogenic activity. It seems now that this requirement is too strict and that some gamma-lactones without double bonds are also carcinogenic. This statement is based on unpublished information [*6*].

Another group of compounds present in heated fat, consists of polymerized material, which has never been proven to be carcinogenic, but Dr. Kummerow here could give a report on how these non-urea adducts may contribute to carcinogenic activity, when fed in combination with certain known carcinogenic agents [*7*].

Many of these compounds could be suspected to be present to a certain extent in the toxic diet, fed to rainbow trout and for that reason, a number of studies were made to determine whether epoxides, hydroperoxides, and/or lactones, were present in specific fractions of the diet which had produced hepatomas in trout. The data will be presented shortly by Dr. Sims, whose group carried out this work. He will also discuss a search for aflatoxin in the particular sample of diet which was supplied to him. After his presentation, I would like to return and discuss a different problem related specifically to aflatoxin. At this time, I would like to introduce Dr. Sims, who will present his data on Analysis of Hepatocarcinogenic Fish Meal Lipids.

[*Dr. Sims' paper was presented at this point. Dr. Falk's paper continues on page 182.*]

ANALYSIS OF HEPATO-CARCINOGENIC FISH MEAL LIPIDS

By R. Sims
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The lipids from meal, provided by Dr. John Halver, Western Fish Nutrition Laboratory, were examined by several techniques in an attempt to identify the causative agent for trout hepatoma. Both a total lipid sample which was very active (50–60 percent liver tumor incidence) and an *alpha* fraction which had been purified by column chromatography on silicic acid (15–20 percent liver tumor incidence, 44 percent by weight of total lipid) were analyzed.

The total lipid fraction had previously been tested by duckling bioassay for aflatoxin at MIT. The bioassay showed some liver damage potential, but was atypical for aflatoxin. The most significant observation made in our study was that no aflatoxin could be detected in either sample.

Solutions (25 percent) in carbon tetrachloride were diluted 1:10 for examination by thin-layer chromatography. A TLC plate coated with silica gel GH (300 μthick) was spotted with 1, 5, and 10 μl of solution as well as with 10 μl (0.006 μg.) of aflatoxin B_1 and mixtures of lipids and aflatoxin B_1. The chromoplate was developed with 7 percent methanol in chloroform. The *alpha* fraction showed a yellow-green fluorescent spot at R_f ca 0.92 while the total lipids gave a yellow fluorescent spot at R_f ca 0.93 with heavy tailing. The R_f for aflatoxin B_1 was ca 0.83.

	EXCITATION λ (mμ)	EMISSION λ (mμ)
A. ALPHA FRACTION		
1. 0.01% IN HEXANE	305	332
	348	383
2. 0.1% IN HEXANE	305	363 (337)
	400	435
	450	515
B. TOTAL LIPIDS		
1. 0.01% IN HEXANE	308	332
	348	383 (445)
	365 (390)	440
	460	515
2. 0.1% IN HEXANE	365 (390)	445
	390	460

FIGURE 107.—Fluorescence of suspect commercial diet extracted lipid.

Fluorescence of the lipids was examined in hexane using a Farrand Spectrofluorimeter (fig. 107). It is apparent that some of the fluorescent spectra are concentration dependent. Also the total lipids sample has one absorption not found in the *alpha* fraction, namely Ex 365(390), Em 445. This absorption lies fairly close to that reported by the Tropical Products Institute for aflatoxin (Ex 365, Em 425). However, emission set at 425 mμ gave only the 390 mμ absorption in the 0.1 percent solution despite a 445 emission with 365 mμ excitation. Thus, aflatoxin was not observed.

By scraping the 0.006 g aflatoxin spot and solvent extracting it, a weak spectrum (Ex 353, Em 423) was obtained, illustrating that aflatoxin can be detected down to about 1 ppb. A total lipid spot containing 0.006 g of added aflatoxin was also scraped and extracted. It showed a weak aflatoxin spectrum but a stronger (5 times) Ex 305, Em 332, which very likely corresponds to the first values shown in figure 107.

Figure 108 shows infrared spectra of the total lipid and the *alpha* fraction. Even after drying over Na_2SO_4, showed OH at 3400 cm^{-1}. Characteristic fat unsaturation was evident at 3000 cm^{-1}. This carbonyl could represent δ-lactone but it should then have a harmonic at 3472 cm^{-1} which is absent or concealed by the hydroxyl absorption.

The curve for the *alpha* fraction showed no OH at 3400 cm^{-1} nor the δ-lactone overtone at 3472 cm^{-1}. The peaks at 1595, 1573, 1115, 1065, and 732 cm^{-1} were all indicative of dioctyl phthalate. This suggests that at some point during the isolation of the *alpha*

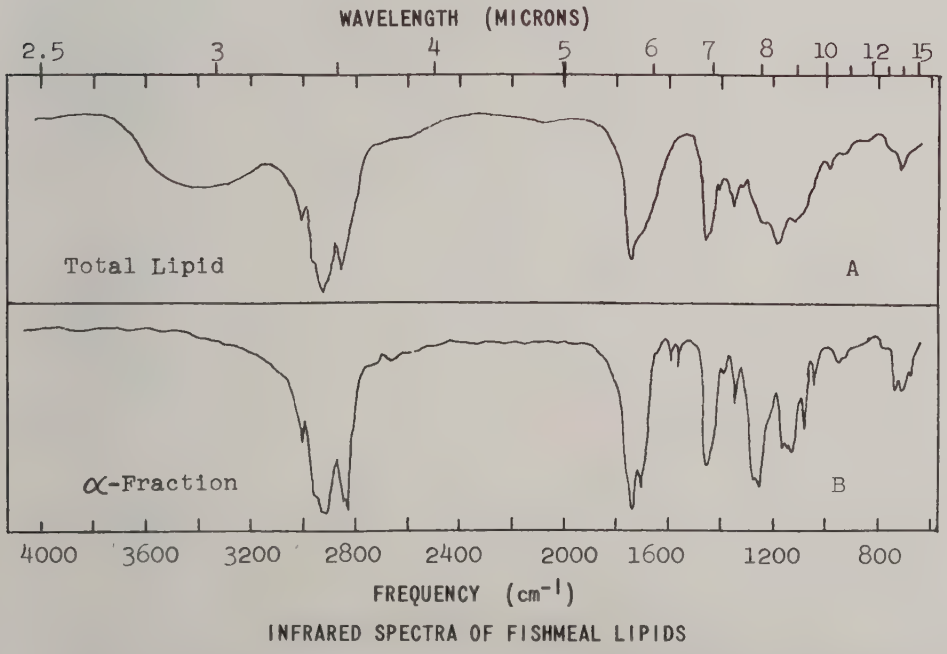

FIGURE 108.—Infrared spectra of suspect commercial diet extracted lipid fractions.

CARBONYL DETERMINATIONS

SAMPLE	WT. IN 5 ml	O.D. 430 mμ	O.D. 460 mμ	MEQ. UNSAT/ 100 g	MEQ. SAT/ 100 g
ALPHA	0.0298	0.380	0.215	NONE	4.34
TOTAL	0.0248	0.760	0.745	2.62	4.68

FIGURE 109.—Carbonyls in extracted lipid fractions.

fraction the material came in contact with Tygon which is plasticized with dioctyl phthalate.

Near infrared and ultraviolet spectra were also run. The ultraviolet spectrum confirmed the suspected presence of α,β-unsaturated carbonyl compounds at 224 mμ. There were little or no hydroperoxides, terminal epoxides, or free carboxyl groups [*1*].

Chemical tests confirmed the spectral analyses. Peroxides were determined by a modified AOCS procedure (Cd 8–53) giving 4 meq/kg for the *alpha* fraction and 22.7 meq/kg for the total lipid which had been exposed to air in transit. Oxirane values were determined using a picrate method developed in our laboratory [*2*]. The total lipid fraction showed 5.4 meq oxirane/100 g while that for the *alpha* fraction was nil. Carbonyls were determined by the Chipault modification of the Henick method [*3*]. Figure 109 shows that these values were also relatively small.

Nuclear magnetic resonance spectra were obtained on both the total lipid and the alpha fraction using the Varian A–60 Instrument. The *alpha* fraction was examined as a 25 percent (W/V) solution in CC_{14}. The integrals were taken of the peak areas (fig. 110). The spectrum was rerun with an expanded amplitude with the area 1.65–3.90 PPM corresponding to 14 H/s. Two may be an isolated methylene or epoxy group

NMR SPECTRUM OF ALPHA FRACTION

δ (PPM)	NUMBER OF DIVISIONS	NUMBER OF H's	EQUIVALENT TO
5.28	9	5	1 METHINE AND TWO DOUBLE BONDS
4.13	8	4	2 GLYCERYL METHYLENES
1.65 - 3.90	26	13 (14)	
1.18 - 1.65	88	44	22 METHYLENES
0.5 - 1.18	36	18	6 METHYLS

FIGURE 110.—Nuclear magnetic resonance spectra of the alpha lipid fraction separated by column chromatography from the total lipid fraction extracted from the suspect commercial ration.

and 12 may be methylenes adjacent to oxygen or to a double bond. On the basis of the number of double bonds (two, estimated from the H/s in the area of 1.65–3.90 PPM) it is hard to explain all the hydrogen in this area. This confirms that the sample is a complex mixture.

A small peak in the aromatic region (about 7.50 PPM) did not seem to be associated with the other peaks and may be an impurity. Based on the infrared evidence for dioctyl phthalate, the NMR integrals indicate the possibility of 30±10 percent dioctyl phthalate. NMR examination of the total lipids showed the same peaks as the *alpha* fraction, but with only a slight aromatic peak.

The lipid mixtures were examined on a Carbowax 20 M capillary column. The samples were injected into the GLC apparatus at 65 and 100°, respectively, for the total lipids and the *alpha* fraction. The solvent was removed and the column was temperature programmed at 2.5°/minute to 180° C. Although the curves were similar, there was a large peak in the total lipid fraction after 108 minutes, whereas nothing appeared after 70 minutes with the *alpha* fraction.

An attempt was made to examine these volatiles in the Bendix Time-of-Flight Mass Spectrometer. The GLC unit attached to the spectrometer has a 200-foot DEGS capillary column which gave no detectable volatiles. Then the lipids were saponified and methylated. Methyl myristate, palmitate, and oleate were identified, but there was also a large group of unidentified esters.

It appears to us that if we are going to do more work in this area, we will need to develop nondestructive fractionation techniques so that we can look at relatively pure compounds rather than these complex mixtures that obviously we are dealing with in our search to find some other causative agent than the aflatoxins for hepatomagenesis.

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Continuation of Dr. Falk's paper

DR. FALK: I have been interested in several different aspects related to aflatoxin. The first inquiry looked into the possibility that the type of fluorescence spectrum which was obtained on thin layer chromatography of the lipid fractions which had produced the hepatomas and had given an unexpected fluorescent spectrum, could be explained by shifts due to changes in solvent. Aflatoxin B_1 is insoluble in hexane, but dissolved in hexane containing 2 percent of fat; aflatoxin, dissolved in such a mixture, showed the same fluorescent spectrum as was obtained from one of the samples of the carcinogenic diet; namely, a maximum at 450 mμ instead of one at 428 or 430 mμ as found in methanol. Adding methanol to the mixture extracts the aflatoxin into the methanol changing the fluorescent maximum to 428 mμ, as expected. Some of these shifts in fluorescence spectrum are solvent-dependent and may at times give wrong clues regarding the identity of the compound.

The problem of stability was next investigated, to see whether aflatoxin B_1 was sufficiently stable under the conditions of analysis or whether extrapolations regarding loss of material would be necessary depending on the length of exposure to visible or ultraviolet light. Quantitation would be most difficult, if under the conditions of measuring fluorescence, some of the fluorescence intensity would be lost at a rapid rate.

Figure 111 shows the formation of decomposition products and their separation by paper chromatography. When the material, applied as a spot on filter paper, was excited by means of weak, long wave ultraviolet radiation, decomposition of a portion of the aflatoxin could be demonstrated. Under the conditions used, it amounted only to a few

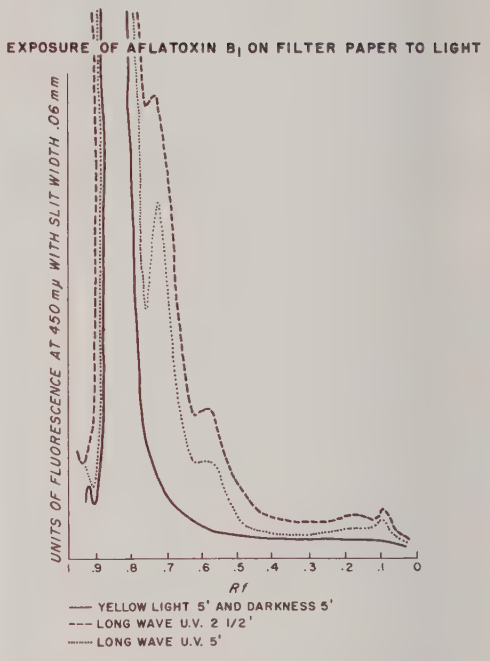
EXPOSURE OF AFLATOXIN B1 ON FILTER PAPER TO LIGHT
UNITS OF FLUORESCENCE AT 450 mμ WITH SLIT WIDTH .06 mm
1 .9 .8 .7 .6 .5 .4 .3 .2 .1 0
Rf
YELLOW LIGHT 5' AND DARKNESS 5'
LONG WAVE U.V. 2 1/2'
LONG WAVE U.V. 5'

FIGURE 111.—The formation of decomposition products of aflatoxin B_1 on exposure to light.

percent, and produced after development, two additional spots with different R_f values, but identical fluorescence spectra. Further studies showed that additional changes occurred, leading to different fluorescence spectra.

The compounds have not been identified, but it looks at this moment that the methods of separation and the measurement of fluorescence will produce partial destruction, depending on the intensity and length of illumination, whether the material is on filter paper, silica gel, or in solution.

Another important problem requiring further study concerned the potential toxicity of aflatoxin to the secondary host. The question was raised whether fish that had been fed aflatoxin, would be toxic to man or the next member in the food chain. Is aflatoxin stored in the body or is it eliminated? If it is stored, where and in what chem-

ical state, or if it is not stored, is it eliminated in toxic or detoxified form? The rate of elimination was also considered of interest.

These experiments were made on aflatoxin B_1 kindly supplied by Dr. Wogan of MIT. Figure 112 shows the rate of elimination of fluorescence in the bile when aflatoxin B_1 was injected intravenously into rats. All experiments were done in one strain, both sexes, and at dose levels varying from 10 to 100 micrograms aflatoxin per hundred grams of rat. The rate of elimination was measured only by the fluorescence recovered in bile and urine. In the early experiments, we did not have radioactive material, so that total recovery of metabolites could not be determined, but only that fraction of the injected material which retained fluorescence could be traced. Judging from the cumulative recovery curve and having heard Dr. Wogan's statements [*8*] regarding the recovery of radioactivity of random labeled aflatoxin, I believe that this recovery represents practically all of the aflatoxin that was administered.

This particular graph represents the recovery after two injections of identical doses of aflatoxin B_1 about 2½ hours apart. We had better recovery and a higher fluorescence intensity after the first injection compared to the 2nd dose, but generally, the liver functioned adequately, even though one might conclude that there was an incipient decrease in efficiency.

Figure 113 shows the fluorescence analysis of three bile samples collected at reasonably rapid intervals, 8, 21, and

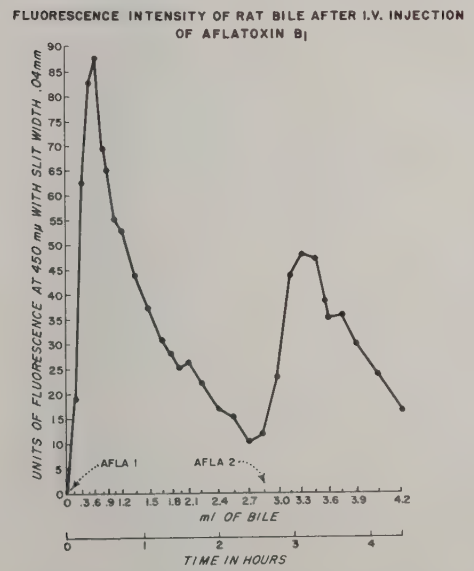
FLUORESCENCE INTENSITY OF RAT BILE AFTER I.V. INJECTION OF AFLATOXIN B1
UNITS OF FLUORESCENCE AT 450 mμ WITH SLIT WIDTH .04mm
AFLA 1
AFLA 2
ml OF BILE
TIME IN HOURS

FIGURE 112.—Recovery of fluorescent products from the bile of rats after i.v. injection of aflatoxin B_1.

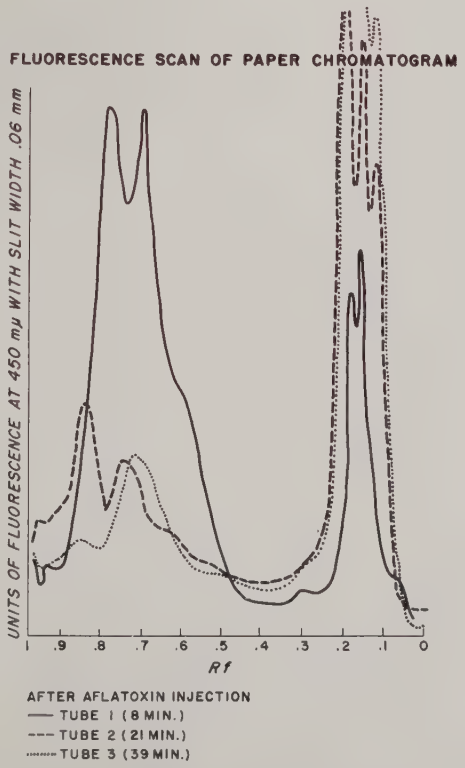
FLUORESCENCE SCAN OF PAPER CHROMATOGRAM
UNITS OF FLUORESCENCE AT 450 mμ WITH SLIT WIDTH .06 mm
Rf
AFTER AFLATOXIN INJECTION
TUBE 1 (8 MIN.)
TUBE 2 (21 MIN.)
TUBE 3 (39 MIN.)

FIGURE 113.—Fluorescence intensity of aflatoxin derivatives separated by paper chromatography, eliminated in the bile during the first 40 minutes after i.v. injection of aflatoxin B_1.

39 minutes after administration of aflatoxin B_1. This chromatogram of the first 8 minute sample shows two peaks with low R_f and then three spots with high R_f values.

The highest R_f value is identical with that of aflatoxin B_1, the next lower ones are those of its decomposition products, while those near the point of application were judged to be conjugates. The nature of the conjugates is still not established, except that no sulfate conjugates seem to be present. However, we have been able to show that as the interval from the injection increased, the free aflatoxin peak declined rapidly, and disappeared completely. Elimination of free aflatoxin and of some of its non-conjugated decomposition products occurs only immediately after injection and the amount of free aflatoxin B_1 in the bile reaches zero 30 minutes after injection, while the total amount of biliary fluorescence reaches its peak at that time, as was shown on figure 112.

The nature of these derivatives has not been established. Based on Dr. Wogan's findings [*8*] that the methyl group of the methoxy phenyl portion of the molecule is hydrolyzed, I assume that one of these low R_f compounds may be a glucuronide, conjugated at the phenolic group that has been liberated by demethylation. Further work is needed to establish this assumption as fact.

It can therefore be concluded that aflatoxin B_1 is not retained to any extent in the rat's body after intravenous injection. Apparently, immediately after injection, a portion is eliminated unchanged in the bile. This represents a very small percentage of the total. About 50 percent is excreted in the bile, apparently as metabolites, and about 40 percent appeared in the urine. Analysis for urinary conjugates of aflatoxin has not been carried out so far, because rat urine contains fluorescent materials, which show fluorescence sufficiently similar to that of aflatoxin and its metabolites to preclude adequate identification of aflatoxin B_1 derivatives. Because biliary fluorescence does not exist in the critical wave length range, fluorescence of aflatoxin and its metabolites in the bile can be followed easily and quantitation can be attempted.

Figure 114 returns to the problem of changes in fluorescence characteristics of biliary aflatoxin and its derivatives that were exposed to light and stored in the dark. Originally it was felt that once the material has been inspected and was placed in the dark, it would remain unchanged. This was found untrue. Some of the compounds changed not only in intensity of fluorescence, but also demonstrated a shift in the fluores-

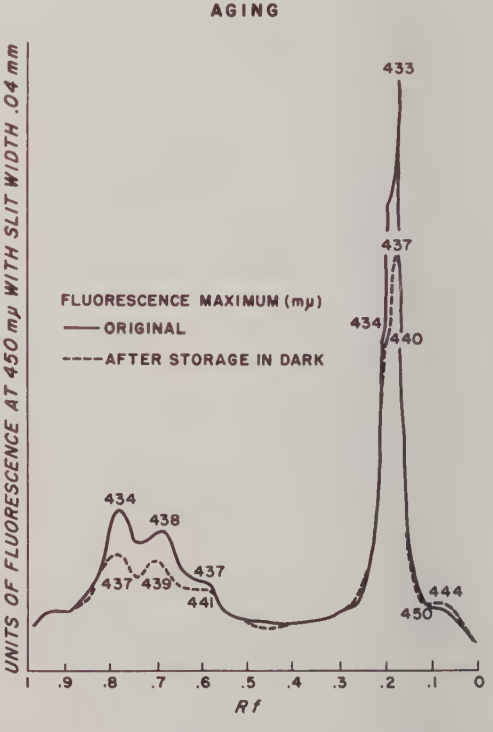

FIGURE 114.—Changes in fluorescence characteristics of aflatoxin derivatives after exposure to light and dark.

cence maximum. A bathochromic shift of the fluorescence maximum was observed on illumination with artificial light, and even on storage in the dark for all aflatoxin metabolites. There is a considerable difference in the rate of destruction, whether one scans a strip under the ordinary artificial light, the ultraviolet light at 365 mμ, or whether one measures fluorescence in an instrument with very high light intensity where one can destroy these compounds in a few seconds unless air can be eliminated.

To conclude, I would like to say that there is definite need for further standardization of fluorescence analysis of these compounds because of the great instability which was found to exist under all conditions which were explored.

Finally it should be mentioned that aflatoxin, when exposed to weak ultraviolet light in solution, produced a product with an absorption maximum at 400 millimicrons, which does not possess any fluorescence. Formation of this unknown nonfluorescent compound will, of course, escape detection by fluorescence analysis.

For individual compounds, no data are available regarding their acute or chronic toxicity. Dr. Verrett of the Food and Drug Administration has kindly tested several bile samples and established the toxicity to chick embryo of an early bile sample containing unchanged aflatoxin, but found none in later bile samples even though very high fluorescence intensity of low R_f components was demonstrated. These findings would indicate that the metabolized conjugates of aflatoxin B_1 are non-toxic to the chick embryo. It cannot establish, however, the lack of toxicity or carcinogenicity of these samples in mammals, where hydrolysis may occur.

I would like to make a comment I think worthy of emphasis for the sake of interesting investigators to extend these findings. Dr. Jackson and his coworker [*9*] wrote a paper a little while ago on the effect of raw liver in the trout diet reversing completely the carcinogenic properties of the cottonseed meal components, thus reducing it to near zero. It would be interesting to see whether there are known components in liver that would counteract the aflatoxin in certain cottonseed meals regarding their carcinogenic response. As Dr. Jackson mentioned, no one at this time was pursuing this matter, and it is worth exploring the possibility of finding antagonists to the carcinogenic action of the aflatoxins or of elucidating interactions between aflatoxins and other dietary constituents.

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Discussion

DR. LA ROCHE: I do not mean to anticipate on the concluding remarks of Dr. Kotin, but since these proceedings might be published eventually, I wish to present my personal conclusions and remarks on what I have heard and read here.

First, I feel that an attempt is still being made to convict cottonseed meal and/or cottonseed press oil as entities. If an adequate demonstration of the indictment had been made, I would be for it. Unfortunately, the work of Mr. Coates shows that some meals may have liver-damaging effects but others may not. Therefore, some cottonseed meals or products should be branded, but not the whole industry.

Now, to discuss a few papers, which pertain to fish exclusively, I would like to start with Dr. Dollar, Dr. Smuckler, and Dr. Simons' venture.

In Dr. Dollar's presentation you have, and I quote, "To identify a specific tumor-inducing agent under these conditions is virtually impossible." I hope that this comment is limited to uncontrolled and uncontrollable situations, because I am positive that the identification of products or combinations of products leading to hepatoma represent the most logical approach to a rational solution. We are after factors which induce cancer in the hope of preventing their appearance in a variety of dietary ingredients, which need not be cottonseed at all.

It is fine to recommend proper storage and refrigeration for dietary components, but still understanding or knowing dietary vectors susceptible of inducing cancer is of prime significance. Lists of precautions yielding uncontaminated dietary components is only an expedient. The identification and mode of action of carcinogens represent the solution.

Later, the best part of this paper comes into phase, and I quote, "It is useful to note that no insecticides or other agricultural chemicals are used in the regions where the pellet ingredients are produced."

This is a very significant point, giving us a fact that something else other than additives may affect liver function and/or morphology.

Finally I would like to comment on our work. From table 43 of Halver's presentation I have isolated the results that are involved. I have just taken a few so as not to confuse the points I wish to make.

The diet pellets gave 29 tumors over 54 animals examined at 16 months. Extracted fat fed at 6 percent of a controlled test diet showed 21 in 51 examined. If this fat is fractionated on silicic acid, through the method described previously, the neutral fat shows only 1 over 48.

According to previous work, the control test diet and the phospholipid fraction have no activity. As we know the total lipid portion induced liver anomalies just as well as the whole diet.

Therefore, silicic acid fractionation either retains the large portion of hepatoma-inducing agents or fractionation has disrupted an essential synergism between neutral and phospholipid fractions. It may very well be that the isolated fractions have lost potency by

chemical alteration on passage through silicic acid. So several possibilities should be kept in mind while this study is pursued.

Knowing that aflatoxin from a crude preparation appears to come off the silicic acid column, with the phospholipid fraction and not the neutral lipid, it would seem probable that the slight activity found in the neutral lipid only, would be associated with something else than aflatoxins. This, of course, will need further confirmation but I do not believe it rash to advance the following: (*a*) aflatoxins induce liver changes; (*b*) some dietary ingredients and only some products found in cottonseed can provoke similar anomalies and (*c*) it is suspected that some dietary lipid products also have hepatomagenic activity.

Finally I would like to say that in fractionating suspected products there is always a danger of inducing or introducing cancerigens which may lead to hepatoma in fish.

DR. DOLLAR: In most hatchery operations it was impossible to trace particular dietary history, source of ingredients, or even the storage conditions of levels of feeding. Hence, except where you have reasonable records of control of your facilities, you have no knowledge of anything regarding the induction of tumors or even their incidence.

DR. HALVER: I don't want anyone to become misled by the ratio of 1 over 48 at 16 months in the neutral lipid fraction fed fish. The same table, shows that at the 12-month inspection, the surgical index of those detectable was 9 over 69. These 9 livers were removed at that time for histological confirmation of the tumors and the remaining fish allowed to continue. Four months later, this additional tumor was encountered. Therefore, fair evaluation, as Dr. La Roche mentioned, would show some activity was consistently found in the neutral lipid fraction. The exact numbers of tumors present at any time-period would be the sum of what was reported here, plus what was removed for histological analysis.

It's very obvious that there is a dramatic loss in activity from the total lipid through the first step of the multi-fractionation.

The other point concerns dioctyl phthalate that Dr. Sims reported in his NMR analysis and other spectrophotometry—we independently encountered this material about the time the sample was sent. We use a large cyclone evaporator designed by Dr. La Roche to concentrate some 30 liters of solvent per 8-hour day, and 3 feet of Tygon tubing was used to pass the solvent into the cyclone evaporator. We removed this tubing and the samples we recently sent should have little in them because we used glass rods plus a very short Tygon connector. We tested a short section of Tygon about this time with the solvent systems used and realized we were not only getting some silicic acids off the columns, but also some ingredients from the Tygon. The Tygon was losing plasticity, we removed it, and we use glass now.

Through the very good and close cooperation of the Department of Agriculture, New Orleans Laboratory, the National Cotton Products Association, and Ralston Purina, we have been able to tie-in what was present in the lipids extractable by Bloor-La Roche technique from the cottonseed meals that Jack Coates has included in the practical feeding study.

Dr. Goldblatt received five different cottonseed meals, which on table 44 are itemized as CSM 1, 2, 3, 4, 5.

Dr. Goldblatt and Dr. La Roche insisted on an anhydrous solvent to re-

move the lipid with the most gentle treatment we could devise at the time using the Bloor rather than the Folch technique. These fats were extracted from the cottonseed meals, we concentrated them, backwashed them to remove water soluble components, reconcentrated them, and then incorporated them in our complete test diet at that level, which would have been present had 20 percent of the original cottonseed meal been included in the pellet.

After 12 months on test we have observed tumors present in these lots of fish. In cottonseed meal fat No. 2 there were 11 tumors seen on surgical inspection. Histology has confirmed these as classical trabecular hepatoma.

The ration that these fish received, contained fat concentrate at 0.69 percent of the diet.

Dr. Goldblatt and Mr. Pons also assayed for aflatoxin load levels in the original cottonseed meal and in the extracted fat. The significant fact was that cottonseed meal No. 2 had about 200 parts per billion of aflatoxin B_1 and some small levels of B_2.

The extract had about 116 parts per billion B_1. This would indicate, of course, that in this particular extract only about one-third of the aflatoxin was removed by the Bloor-La Roche solvent technique; that large portions of this were destroyed or were lost in the water backwash, which we put quotes around and call "carbohydrate," for lack of a better name. We have these concentrates. We also have the residue. The residue was also analyzed and showed that about one-third of the aflatoxin had been removed; that the Bloor-La Roche technique was very inefficient for removing aflatoxin. And if one extends down the level fed, the 0.69 percent times the extract, B_1 content (116 ppb) we end up with 0.8 part per billion B_1 in the ration, which is about the order of magnitude one would expect for 10 to 20 percent incidence of tumors appearing in 1 year.

The point I wish to make is that some lack of progress occurred because we extracted our original suspect ration with a solvent system that would handle the fats gently. We have been very careful about preserving the fat from additional oxidation, therefore, we didn't get much of the aflatoxin out, and under these conditions the results obtained were on the same order of magnitude one would expect with this aflatoxin level in the trout diet.

The other thing that we didn't include in the discussion except under code in table 44 was Dr. Kummerow's polymerized fat with and without small amounts of a known rainbow trout chemical carcinogen. This treatment did respond with a small incidence of tumors. We don't know the final effect yet. We intend to pursue this study, but obviously there is some carcinogenic vector in either the polymerized fat, which was furnished by Dr. Kummerow, or that of the commercially available polymerized fat, under code CFP. You will note that when CFP is incorporated into the diet at 1 percent of the ration, seven tumors in one hundred and eleven fish examined were observed after 1 year on study.

This reemphasizes that livers of these trout are rather sensitive, and can be rather easily insulted by some of these strange and different compounds that may precondition them to triggering action from some very small amount of potent carcinogen. The aflatoxin concentrate which we use as an additive to the diets as they are prepared at biweekly intervals has been stored since the time of the start of the experiment. The aflatoxin has been dissolved and added to alpha cellulose flour. The whole mixture is homogenized care-

fully, the solvent allowed to evaporate overnight, and this dry aflatoxin-alpha cellulose mixture has been the material we have added as part of the diet during the biweekly mixing processes. This material has been stored dry, cold and away from light since the time that we mixed it a year ago.

One more comment. We conducted a cooperative study with the Millers at Wisconsin where 2-acetylaminofluorene, a well established carcinogen for rodents was when fed to fish, negative or practically negative. A very low incidence of tumors occurred. The Weisbergers, Dr. Falk's group and the Millers have shown that one of the early steps necessary to potentiate 2 AAF into an active carcinogen is a hydroxylation of the nitrogen, and the N-hydroxy AAF is the potent carcinogen for the rodent.

To study whether fish had this N hydroxylase present we held larger rainbow trout in a metabolism chamber, cannulated them and collected separately the urine and the feces after force feeding them various amounts of 2 AAF or N-hydroxy AAF.

The Millers have found from these metabolic samples in the urine, in the blood, and in the feces, reasonable quantities of five hydroxy AAF, the seven hydroxy AAF, but no detectable amounts of the N-hydroxy AAF in any of the samples examined. They have looked at three or four experiments, each experiment consisting of from six to ten fish. The 2 AAF has been force fed in the diet, has been injected in various solvent systems, and actually has been implanted surgically by a slit in the abdominal wall. Again, five hydroxy and seven hydroxy AAF was easily detected, no N-hydroxy AAF appeared unless N-hydroxy itself was added, and in this case it appeared in the urine and in the blood.

Thus one small bit of evidence shows that one enzyme system for detoxification is absent in trout liver. We can expect to find and encounter similar effects as we study the metabolism of aflatoxins and the metabolism of these other low-responding carcinogens.

DR. BUHLER: Brodie at the NIH a few years ago reported (Fed. Proc. *17*, 370, 1958) that fish do not have the drug metabolizing enzymes in the liver, using the classical N-demethylation, hydroxylation, and so on, tests for activity.

DR. LEE: It's very obvious that the rainbow trout liver is susceptible to a variety of carcinogens as well as pure aflatoxin, that it is one of the most sensitive test organisms for testing carcinogens; and I know that some of the earlier experiments supposedly ruled out the possibility of there being an infectious agent in these trout. No one observed inclusion bodies that suggested the possible presence of viruses. But in looking at some livers at the New York Aquarium with Dr. Ross Nigrelli, we did turn up a few under special circumstances. There were some trout from Wyoming that had been captured in the wild, estimated 3 years old. These trout developed tumors, but in some of the livers in which no tumors appeared, we did find changes that led us to wonder whether there might not be some other factor present also.

One of the things that makes this observation important is that there is work coming out all the time now; for instance, some of the work of the Demeyers, that some of the carcinogens inhibit interferon production, and allow viruses to be expressed which might otherwise be latent within an organism. You know the classical work of Rouse on carcinogens accentuating the effects of various tumor viruses.

I am not saying that these are virus

inclusion bodies even, because we have not been able to do the experimental work. These were livers that were sent into the aquarium that we went back and reviewed, and actually didn't even spot the inclusions until last year, when we went back and were reviewing some of the old material. But I would like to see what comments other people might have to make about them.

These livers were quite hyperplastic and contained some very typical inclusions. These were intranuclear; and eosinophilic. They do not form within the nucleolus. They invariably displace the nucleolus to one side, and I think in a large series of these that that's fairly obvious. They are not very common, but often occur in clusters within a liver.

At times these nuclei eventually seem to disintegrate, and these particles can sometimes—or what I think are probably particles, seem to be discharged.

One can see gigantic nucleolar changes that take place in many of these cells, but when the inclusion body forms it displaces the nucleolus to one side. It does not appear to form within the nucleolus itself.

The staining is also different from the nucleolus, which usually takes up a red element from the Picro-Mallory stain, whereas the inclusion body itself stains much more lightly.

Has anyone else seen any of these things? Doesn't it suggest that perhaps we cannot rule out an infectious basis for hepatoma? It reminds me of the situation with Aleutian mink disease, where for a long time the etiology of this disease was not known. It was thought to be a genetic disease specific to the Aleutian mink, and there is no doubt that the virus, which has now been pretty well demonstrated in the Aleutian mink disease, may be predisposed to affect the Aleutian mink. Nevertheless, at this point it's been found also to be infectious for many other strains. Maybe this explains why, in the early days we weren't able to produce tumors in the eastern brook trout, but now tumors are appearing in the eastern brook trout.

DR. WOLF: Was there any pancreatic tissue along with your liver material? Sometimes there is a little thread of it that will hang up on the back side near the bile connection, and if you can find any of this, I suggest you take a good look at it.

DR. ASHLEY: We have seen similar inclusions, eosinophilic, in occasional trout livers that we have studied for hepatoma. We have been unable to do any serious histochemical work on them, for lack of time, but they look very much the same.

DR. LEE: Certainly in many latent viruses the virus may not be found at the time when its effects are being produced in the animal. I know even with the Lucate frog carcinoma people are still arguing about that, although I don't think there is much doubt but that the virus plays an important role in the development of the tumor. There, the inclusion bodies can only be found at certain times. In fact, they are only found after the frogs have been kept at a very low temperature, and just at the time the tumors are beginning to appear at low temperature is when the inclusions appear. Later they disappear completely.

DR. GOODALL: I suggest the observation of Dr. Lee is really very worthwhile. I only want to remind you that this business of inclusions doesn't necessarily imply viral infection. In other species, anyway it may occur from metal poisoning, lead, maybe zinc. I know from some of my own unpub-

lished data, chronic overdosage of cortisone also produces occasionally internuclear lesions similar to the ones you showed.

DR. LEIBOVITZ: I wonder why no one brought up tissue culture in these discussions. At the beginning of this study it was obvious that everybody had the entire problem to attack, but now we are getting down to definitive chemicals and definitive diagnosis as to the possible cause of these hepatomas. Tissue culture could be a very sensitive and readily controlled tool for study of aflatoxin, possible virus infection, and many other problems, instead of trying to do all this in an entire trout. I realize it's a new discipline to impose upon the group, but it may give many of the answers and less time than the year required for trout to develop a hepatoma.

MR. SINNHUBER: We have a man on our staff, Dr. Fryer who has been successful in culturing trout hepatoma tissue for 18 or more transfers. We have seen some very unusual inclusion bodies with the electron microscope in this tissue cultured material. I would appreciate some comments on just how valuable this might be as a tool for this trout study. We would like very much to be able to culture "normal" liver, trout liver.

DR. SCARPELLI: We tried and we got a cell that we are really not sure is a liver cell. It's fibroblastic in appearance and elongated and just doesn't have any of the characteristics morphologically of a hepatocyte.

DR. HALVER: A fibroblast isn't a normal liver cell.

DR. LEIBOVITZ: It is usual in tissue culture work, no matter what organ you start with, to end up on serial passage with a fibroblast as the residual "normal" cell. Most of the epithelial-like cells that are serially passed in the laboratory are malignant or abnormal cells. Fibroblasts isolated from different human embryonic tissues are quite different, and are characteristic for the organ from which they were originally isolated, at least while they remain as theoretical "normal" cells or "diploid" cells, as determined by chromosome studies. So even though every organ is a mixture of cells, the fibroblast appears to take over. However, you may get many answers from the fibroblast derived from liver instead of needing the entire organ.

DR. KOTIN: Do you mean that in fibroblasts grown out of liver, you can get inferential information for the parenchymal cells of the liver?

DR. LEIBOVITZ: I don't mean from a biochemical histological aspect, I mean on susceptibility to virus agents. I am primarily talking about virus isolation and identification studies here.

DR. KOTIN: There is always the calculated risk that as of right now we really have no profile of the native viral population of the cell line. This truism is a matter of common knowledge due to the complicating factor of things like organisms et cetera, which are becoming more and more astounding in terms of existence in cells of diverse types.

DR. LEIBOVITZ: There are certain chemical tests that can be done to determine the relation of the isolated cell to the original organ—for example, arginase or other specific enzymatic activity.

DR. EVELYN: In this connection, I recently spoke to Dr. John Fryer, Oregon State University. He has not had much luck in growing normal fish parenchymal liver cells but was able to grow with difficulty neoplastic type liver cells. The latter had prolonged

generation times, however. Dr. Fryer prefers to use undifferentiated cells obtained from fertilized salmon eggs since they are propagated much more readily and are susceptible to the viral disease he is studying. It may be that such cells would prove satisfactory for tests on aflatoxin.

SYNOPSIS

By Paul Kotin, *Scientific Director for Etiology*
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DR. KOTIN: First, let me say that the title of the workbook, in the light of the deliberations that have taken place, appears to be something of a misnomer, for this has been essentially three conferences in one. It has been a conference on aflatoxin as a food contaminant, on carcinogenesis as a biological phenomenon, and on hepatocellular carcinogenesis as a specific entity. I might add that in many ways it also has been a conference on public health.

I would be remiss if I did not comment upon the fine cooperation that has evolved between industry's researchers on the one hand and researchers in government and academic institutions on the other. All have worked harmoniously to develop a full exchange of information on the aflatoxin problem, to the benefit of the public. The difference in governmental responsibilities for public health, as compared with those of other groups, relate to the various missions of government units, both State and Federal. The National Institutes of Health, because it is a nonregulatory agency, can operate within dimensions that are quite different from those of the Food and Drug Administration. Fortunately, however, the researchers in the Food and Drug Administration maintain the broadest possible horizon, and do not limit themselves simply to the recognition and definition of a problem. Wherever possible, in terms of available manpower and facilities, that organization attempts also to find solutions to problems affecting the health of the public. Unfortunately, from time to time a distorted view of the purposes and methods of the Food and Drug Administration has been projected. Let me assure you that that group has worked diligently to solve the problems pertaining to pesticides, cigarettes, food additives, drugs, and vaccines, and now aflatoxin.

Although we may succeed in controlling to a high degree the conditions of experimentation, there remains the very important problem of interpretation of morphologic pathology. The elegant descriptions of pathology by Drs. Simon, Ashley, Newberne, Wales, Yasutake, and others have been individually informative. However, their descriptions placed side by side draw one's attention inescapably to a need for standardization of terms to render them more meaningful. Their presentations only too well demonstrate deficiencies in microscopic pathology as it is practiced today. The descriptions of marginal pathologic changes in that gray zone between normal and abnormal are limited by the now rather gross, imprecise nature of light microscopic pathology. To say it more briefly, the secrets we seek lie beyond the resolution of the light microscope. Let me amplify this somewhat further: It is doubtful if a difference of opinion will arise between two pathologists seeking to distinguish between a carcinoma of a liver and a normal liver; but the characterization and the future significance of a nodule is another question. The pathological study of the liver is especially difficult because nature has failed

to provide some of the clear demarcations between the normal and abnormal easily recognizable in other organs. The British have taxed nearly to the limit my anglophilic tendencies by initiating and popularizing the use of the term "hepatoma," for, strictly speaking, it simply means a tumor of the liver, and this term is capable of covering a multitude of lesions, as has been amply demonstrated in this and many other conferences.

Let me turn now to the substance of the conference. One of the most pressing needs resulting from these discussions is a crystallization of the theoretical and practical facets of experimental pathology. It is amply evident that standardization of conditions of experimentation is urgently needed. I do not believe it would be an overstatement to say that standardization of conditions employed in cancer research, both carcinogenesis and chemotherapy, over the past decade has contributed as much as any other single factor, and perhaps all other factors, to the progress that has been made. Without such standardization, the volume of work required to generate meaningful data is increased manyfold, particularly where the data are derived from studies conducted in separate laboratories. Perhaps the experimental model in greatest need for future studies of trout hepatoma is an isogenetic strain of fish. Other factors familiar to the ichthyologist and worthy of the closest possible control include the ages at which the animals are exposed to potential carcinogens and other toxic materials, and such environmental factors as water temperature. The age of the experimental animal has been demonstrated to be singularly important in a variety of carcinogenesis experiments involving newborn mice and rats. Indeed, with respect to the same parameters, there may be greater similarity between a newborn mouse and a newborn rat than between a newborn mouse and an old mouse. For an animal that is poikilothermic it seems most reasonable to expect response to varieties of stimuli to be influenced by the ambient temperature.

While I am discussing the subject of morphology, I wish to direct a few remarks to comparative pathology. Our experience with human pathology has taught us to lean heavily on such characteristics of neoplasms as the development of metastases, the invasion of blood and lymphatic vessels, and the ability ultimately to kill the host. When we attempt to apply some of these characteristics to other species, we sometimes fail to recognize that they do not fully apply. For reasons that are best known to a mouse, that species is peculiarly deficient in developing metastases from its primary cancers, all other things being equal. For this reason, the ability of a tumor to metastasize is not necessarily a reliable index of its malignancy unless one defines the species. Similarly, the carcinogenicity of a substance or set of conditions may vary from species to species, and can be defined only in terms of individual species. To pursue this matter one step further, the natural history of the liver nodule most probably differs from species to species, and perhaps even from one strain to another within a species. It is for this reason that isogenetic strains of trout will be needed in the definition of the natural history of the liver nodule. Parenthetically, it would be most interesting to take a page from the book of Dr. Jacob Furth, who was the first to perform isologous transplants of organs and parts of organs to the spleen, in animals. Adoption of

this technique for studies of eosinophilic and basophilic nodules of the liver might help us to define the significance and natural history of such nodules in trout.

During this conference, attempts have been made to distinguish between cholangiolar and hepatocellular carcinomas and between adenomas of the biliary ducts and adenomas of the hepatic parenchymal cells. In addition, there was some question whether the increased concentration of tubules seen in these livers represented central degeneration of nests of liver cells or were primarily of biliary duct origin. To me it is questionable that discussions of this nature will advance our understanding of the problem significantly, for the bile duct epithelium and the liver parenchymal cells have a common embryologic cell of origin; and the frequent similarities of histologic patterns should not surprise us if we accept the popular concept of neoplastic disease as a reversion phenomenon.

Let us not be misled, too, by the biphasic cellular patterns seen in these liver tumors. This is a phenomenon not limited to aflatoxin-induced hepatic disease but is characteristic, for example, of the two populations of cells described in ethionine-induced tumors of the liver. I do not believe anyone would question that the two or possibly more populations of cells may well have arisen from common progenitor cells. What is important is that hepatocellular carcinomas, in terms of their natural histories within a given species, behave rather consistently as a group.

There has been much discussion of the significance of lipids, fatty metamorphosis, ceroid, and glycogen storage. I believe care should be taken to avoid attributing specific etiologic significance to variations of lipid content and morphology and to disturbances of glycogen storage and the presence of ceroid, for these abnormalities almost certainly have a multiplicity of origins. Rather, they should be regarded as significant indications of disturbance.

As much as the pathologist would find it comforting to be able to associate specific histologic appearances with identifiable etiologic agents, in individual microscopic slides, that is possible only rarely. For this and other reasons, while it is imperative to continue studies of morphologic pathology, we must extend our research to pathology at the molecular level. In the long run, this may well be the shortest way home.

From time to time, in our frustration over the long time needed for carcinogen bioassay, we have sought to develop new and more rapid means of detecting carcinogenic potential of chemical compounds. We have attempted to devise quicky tests with biological end points other than the development of a tumor. All such efforts have been without success. I venture to predict that we shall successfully elucidate the mechanisms of carcinogenesis via the molecular studies route, thus obviating the need for such quicky tests. In brief, I believe the molecular approach to carcinogenesis and the development of quicky tests dependent upon nontumorous endpoints are mutually exclusive concepts, since by the time we have a firm basis for rapid tests, our knowledge of carcinogenesis will be so fundamental as to permit precise carcinogen identification.

The role of diet in the pathogenesis of the acute toxic changes and the neoplastic response has occupied much of our attention. The data of Wolf and his coworkers relating carcinogenicity to the cottonseed meal components of the

diet are truly impressive. However, the question remains: Is a component of the meal itself carcinogenic, or is the meal but a vehicle for a contaminant with carcinogenic activity? The evidence to date points very strongly to a contaminant, aflatoxin, as the etiologic agent in trout hepatoma. I should caution, however, that our experience in cancer induction in general should not be ignored, for it may be true in the case of trout hepatoma as in many other cancers that the interaction of two or more factors may be needed to effect conversion from the normal state to cancer. This is not to ignore the possibility that the diet, indeed the cottonseed meal *per se*, may contain one of the factors while also acting as a vehicle for another, specifically aflatoxin. And while mentioning the interaction of multiple factors, one should not neglect to consider the possible role of viral agents in oncogenesis.

At this point we should recognize that the simple identification of one or more of the etiologic factors is but a first of many goals that must be achieved. The fact that aflatoxin, a natural product capable of contaminating many human food resources, is a potent carcinogen, at least for animals, dictates that its mechanisms of action be explored fully. The works of Drs. Scarpelli, Novelli, and Wogan, separately, elegantly demonstrate the benefits to be derived from the applications of multiple and varied disciplines to this problem. I believe the electron microscopic studies, particularly as they may be utilized by the biochemists for comparing alterations in ultrastructure with alterations in biochemical behavior, have implications of tremendous importance not only for carcinogenesis but also for acute toxicity. Those disciplines concerned with immunology have done much to exploit the special susceptibility of the very young and newborn members of a species in contrast to the adult and aged members of the same species. A most impressive case can be made for the state of immunologic competence of the host as being critical in determining the susceptibility or relative refractoriness to the action of carcinogenic materials. To offer my own prejudice on this matter, I should say that possibly ribosomal and enzymatic differences that exist among the newborn, the young, and the adult *cells* provide an equally valid theoretical explanation for the differences in host susceptibility. The whole concept of enzyme induction as the basis for detoxification of carcinogens and other noxious agents is one that receives much support at this time. However, as Dr. Halver points out with respect to the aromatic amines and the azo dyes, some of these detoxifying enzymic activities seem almost to be suicidal in their intent, for they convert, in some instances, noncarcinogenic chemicals to their carcinogenic form through a process of N-hydroxylation.

The portion of the molecule responsible for hepatoxicity is not necessarily that responsible for the carcinogenicity. So far as I am aware, there is little or no evidence to support the assumption that both follow the action of a single submolecular fraction. Some of the work performed in the laboratory at the Chicago Medical School with respect to acute toxicity of carcinogenic hydrocarbons would indicate that it is reasonable to suppose that carcinogenicity may reside in a different portion of the molecule, as in the case of the polynuclear hydrocarbons. It is important to recognize the possible existence of differences between the chemical structure of the proximate form of the

agent and its structure as it occurs in the environment. These considerations render especially important Professor Buchi's attempts to synthesize pure aflatoxin. In the course of synthesis of the aflatoxin molecule, as it is being built up, it will be possible to perform bioassay studies to identify those components of the molecule which confer toxicity on the one hand and carcinogenicity on the other. Such studies will provide us with invaluable information on the relationship of structure to function, and might be expected to clarify to some degree the relationship of acute toxicity to carcinogenicity.

To return to the problems involved in comparative pathology of aflatoxicosis, to which Dr. Newberne has contributed significantly, let us be reminded of some critical interspecies differences that may cause confusion. The early work on experimental induction of bladder tumors with beta-naphthylamine was long frustrated by failure to employ the appropriate animal species. Long before it was known that N-hydroxylation was an essential to carcinogenic action by this material, and before it was realized that the guinea pig, for example, is incapable of effecting N-hydroxylation, much time and effort were expended attempting to achieve the unattainable. Finally, Hueper and Wiley in their experiments utilized dogs, which are capable of effecting N-hydroxylation, and thus succeeded in developing an experimental system for bladder tumor induction. If bioassay were limited to mice or guinea pigs, the role of this compound in bladder carcinogenesis would be questionable. And I would remind the group that interspecies differences may be complicated even further by the differences that exist in metabolic systems from one age group to another in a given species. The whole matter of substrate induction of enzymes is keyed closely to age of the host. It is known, for instance, that the liver of the newborn mouse is relatively refractory to enzyme induction by drugs; in my experience, the same observation applies to carcinogenic hydrocarbons.

Before closing this conference, we should at least recognize the need to define the "spontaneous" occurrence of hepatoma in trout. Without such information we shall be seriously hampered in our efforts to determine the significance of experimentally induced tumors in trout. Unfortunately, this need cannot be met satisfactorily until isogenetic strains are available. I should predict that genetically defined strains of trout will contribute as much to the progress in this area as have the elegant procedures for analysis and quantitation of lipid constituents of the diets, plus aflatoxin contamination of the diets. Clearly, however, each of these factors makes its contribution and must be exploited fully.

A logical extension of the consideration of interspecies differences is the question of relevance of cancer-induction experiments in animals to the etiology and pathogenesis of cancer in man. This problem has been so formidable as to discourage pursuit by the scientific community, on the whole; and yet it is one with which we must eventually come to terms, because experimentation of this nature in man is clearly unthinkable. I suggest that although the total answer may not be obtained by correlative animal experimentation and epidemiologic studies in man, nonetheless a highly significant body of information could be developed by these methods to make extrapolation from several species of animal to man much less hazardous.

The epidemiologist is compelled to study human populations in their natural settings and, therefore, may be obliged, for the purposes of studying primary hepatic carcinoma, to travel beyond the confines of the United States to such areas as West and South Africa. There we begin to see relationships that tend to credit the significance of aflatoxin to man. The tropical and subtropical areas provide a most favorable environment for luxurious growth of molds. The peoples of some of these areas, by virtue of their ecological and cultural circumstances, fail to distinguish clearly between the foods and the contaminants of foods. Interestingly, in certain parts of West Africa, the moldy corn is considered a delicacy. The temptation to explore this matter and its relationship to the causation of liver cancer in man is almost irresistible. Unfortunately, epidemiological studies depend for their success upon the availability of meaningful demographic data and competent epidemiologic skills, and these are rarely to be found nor are they easily developed in these areas.

The difficulties of extrapolation from animal systems to the human problem should be faced squarely whenever the temptation arises to pursue the development of a new bioassay system such as has been suggested from time to time, and in particular during this conference. Admittedly, the fish hepatoma would provide a very sensitive system, and this might have real value in studying the mechanisms of carcinogenesis, particularly in relation to the structural components bestowing carcinogenicity on a variety of chemical substances. The biochemistry of hepatic function in the fish suggests explanations for its marked sensitivity to certain types of agents and rather complete insensitivity to others, including 2–AAF and related compounds. But this extreme sensitivity is not without its problems, for it increases greatly the difficulties of extrapolating the results to man. Extrapolation is hampered by at least several factors, not the least of which is the failure to know whether there is a threshold for response to a carcinogen. This is particularly important if attempts are to be made to extrapolate where quantitation may be significant in determining responses.

A few remarks would be in order to place studies we have been discussing in perspective in relation to the spectrum of past and ongoing carcinogenesis research. First, let me emphasize that research over the past two decades has firmly established the importance of interaction of multiple factors in carcinogenesis. This has not simplified the tasks of those who must clarify carcinogenesis in man, for he is exposed to an infinite variety of potential carcinogens in widely varying doses throughout his lifetime. For example, molds are most ubiquitous coinhabitants of man's environment. One such mold, *Aspergillus flavus* (Link), has been demonstrated, as we know, to produce a most potent metabolic product that has been proven to be carcinogenic for some animal species. It would be naive to assume that *Aspergillus flavus* (Link) is unique amongst molds in its endowment with this capability. The day is not far off when attention will be turned to carcinogenic potential of a host of cousins to *Aspergillus flavus*, including *Penicillium*. Certainly the findings with respect to *Aspergillus flavus* compel the attention of Government agencies and industry, but the attention must take the form of multidisciplinary research to identify, define, and characterize the carcinogenic potential of natural products. If the disciplines represented at

this conference will continue to collaborate as they have in the past, I foresee a significant acceleration of research in this area.

Finally, in closing, I wish to compliment Dr. Halver and Dr. Mitchell for bringing together a group with such diverse yet harmonious interests and talents, and to thank all of the speakers and attendees for their participation in this important work.

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